Dissertation

Fermentative Production Process of 2-pyrone 4,6-dicarboxylic Acid from Different Biomass Feedstocks

Graduate School of
Natural Science and Technology
Kanazawa University

Division of Natural System

Student ID No. : 1524062003
Name : April Nway Nway Htet
Chief advisor : Prof. Kazuaki NINOMIYA
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Preface

This research was conducted under the supervision of Prof. Kazuaki Ninomiya at Department of Biotechnology, Institute of Science and Engineering, Kanazawa University, from April 2015 to March 2018.

The objective of the present study is to extend the microbial process for 2-pyrone 4,6-dicarboxylic acid (PDC) production by using biomass feedstocks. The author hopes that the investigations would support to produce PDC effectively and economically by recombinant *Escherichia coli* (*E. coli*).

April Nway Nway Htet

Department of Biotechnology

Graduate School of Natural Science and Technology

Kanazawa University

Kanazawa 920-1191, Japan

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General Introduction

Fossil resources are generally used to produce many important chemicals [1]. Essential parts of modern life associated with human daily life are made using petrochemicals [2-3]. Approximately 7 % of the global petroleum production was used for plastics production. For example, fuel consumption vehicles and aircrafts can be reduced using light-weight plastic components [3]. Over-consumption of fossil resources generates the negative environmental impact which could lead to global warming and climate change [4]. Some of the petrochemical-based materials are shown in **Table 1** [5]. To reduce the dependency on fossil resources and greenhouse gases emission, alternative carbon resources are imperative and implemented [1, 4]. Like petroleum feedstock, biomass feedstock has a complex composition and needs treatment for conversion into various products. Thermal stability of biomass is lower than petroleum, but it has higher functionality [6]. The worldwide application of biomass in 2008 is shown in **Fig. 1**. The characteristics and performance of bio-based plastics are comparable to petroleum-based plastics [7].

Bio-based products are mainly derived from the oleochemical industry. Biodiesel is mainly produced from plant oil via transesterification of triglyceride and methanol. Glycerol is the by-product of biodiesel production. It can be applied to a wide range. In 2009, the demand for glycerol was 1.8 million tonnes. Some of the chemicals (e.g. 1,3-propanediol, acrylic acid) derived from glycerol have received particular interest [8]. For biodiesel production, it is beneficial for integrating by-product glycerol conversion into the value-added products. But plant oil is edible food and the cultivation competes with the other agricultural crops.

The other potential candidate that can convert into the high-value products (e.g. biofuel) is lignocellulosic biomass such as bagasse [9]. Bagasse can be obtained as a

Table 1 Examples of petrochemical-based materials [5]

Petrochemical products	Uses
Polyester, nylon	Textiles for clothing and carpets
Polyurethane	Adhesives and resins for particle board
PET	Lightweight, unbreakable bottles
Polyethylene	Plastic bags
Expanded polystyrene	Packaging for fragile goods
Various high-grade plastics	Artificial limbs, spectacle lenses, crash helmets
uPVC	Doors, window frames
Glycols	Surfactants for basic soaps and expensive cosmetics
Ammonia-based fertilizers	Increasing crop yield
Insecticides and biocides	Health protection
Plastic composites	Lightweight vehicles
Synthetic rubber	Tyres

PET : polyethylene terephthalate

uPVC : unplasticized polyvinyl chloride

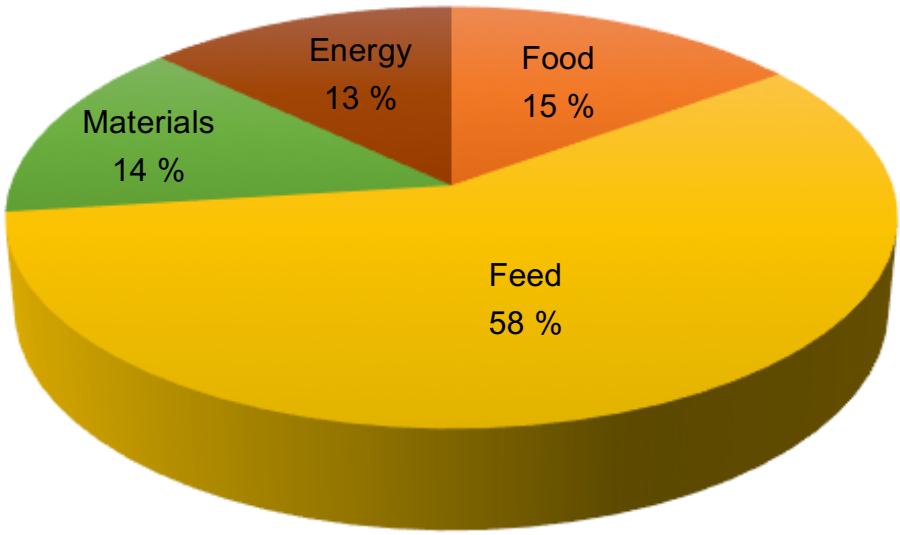

Fig. 1 Global application of biomass in 2008 (13 billion tonnes biomass) [7]

waste material after extraction for sugar production. No additional land used is required to obtain bagasse. Generally, the lignocellulosic feedstock is composed of cellulose (40-50 %), hemicellulose (25-30 %), lignin (15-20 %), and trace components. The monosaccharide (glucose) derived from cellulose and monosaccharides (xylose, arabinose, mannose, and galactose) derived from hemicellulose can be used as substrates for ethanol production by fermentation. The crystallinity and rigidity of lignocellulose structure must be broken down to convert embedded polysaccharides into fermentable sugars [10, 11]. Therefore, pretreatment process of lignocellulosic biomass is the important and the pretreatment method used effects on the downstream processing. The obtained fermentable sugars from pretreated lignocellulosic biomass can be used for the production of fuels or chemicals (e.g. bioethanol, lactic acid, etc.) [7, 12].

Apart from land-based feedstocks, algae (macroalgae and microalgae) can be used as a biomass source. Algae (more than 40,000 species) that grow in fresh or salt water

use the sunlight as an energy source for the conversion of CO_2, water, and inorganic salts into the biomolecules [5, 7]. Comparing with land plants, the productivity of algae is high, and no arable land is needed for algae cultivation. Therefore, it is no competition with agricultural crops. Algae use sunlight, water, CO_2, and inorganic nutrients (carbon and nitrogen) for the growth. Carbon and nitrogen sources can be obtained from waste discharge [7]. Furthermore, algae biomass can be harvested at any time and contain no lignin. The main composition of algae can vary depending on the species. The main energy reserves in algae are starch and lipids under nutrient starvation and variation in growth conditions [11, 13]. Microalgae use as an alternative feedstock for the production of biofuel (e.g. biodiesel and bioethanol) [14]. The general illustration of glycerol, bagasse, and microalgae can be seen in **Fig. 2**.

Nowadays, the annual fermentation products are approximately 8 million tonnes [8]. The natural products, biomaterials, are biologically synthesized and catabolized by various organisms and applied for a wide range such as medical application [15, 16]. The development of genetic and metabolic engineering supports to access the potential industrially biosynthesized of chemical building blocks such as succinic, itaconic, and glutamic acids in recombinant organisms [17, 18]. Petrochemical cannot be used as raw material to produce some building blocks such as lactic acid and 2-pyrone 4,6-dicarboxylic acid (PDC) [7, 19].

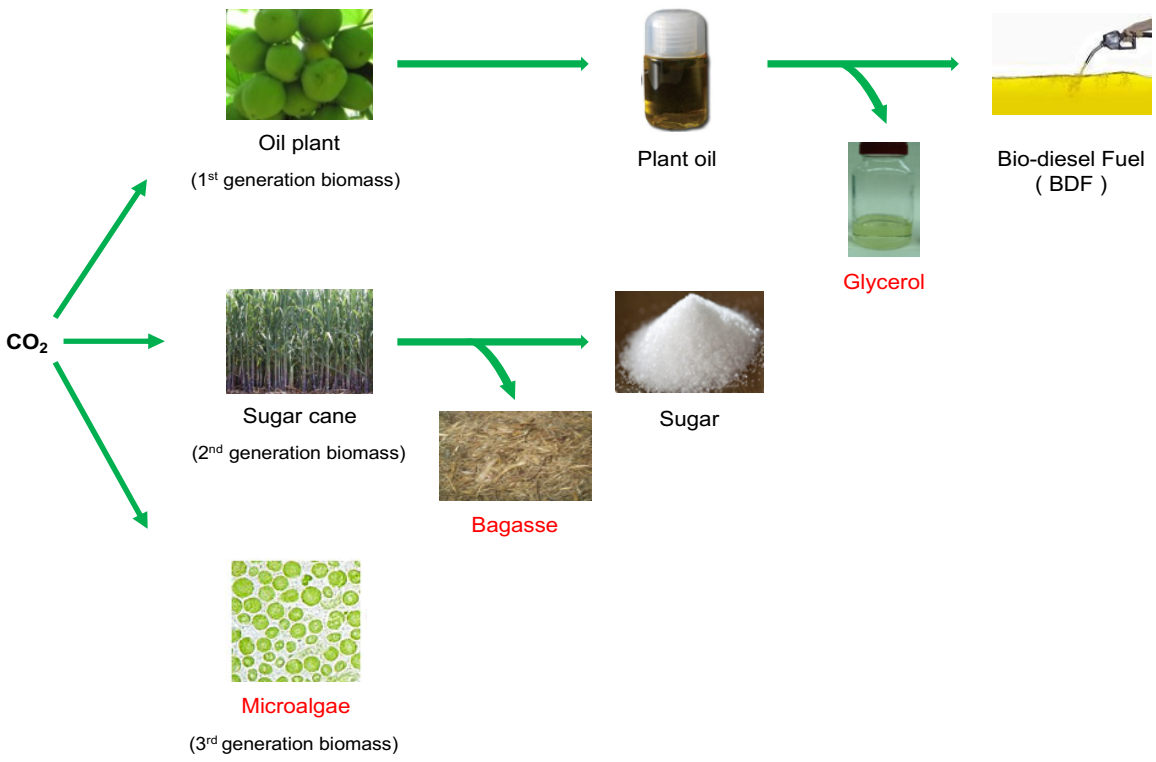

Fig. 2 The general illustration for glycerol, bagasse, and microalgae

2-pyrone 4,6-dicarboxylic acid (PDC) is a dicarboxylic acid with a polar pseudo-aromatic ring with the molecular weight of 184.1. It has a similar molecular shape to isophthalic acid, which is currently used for the synthesis of various kinds of polyesters and polyamides, such as polybenzimidazole; therefore, as a monomer, PDC has the possibility of producing high-performance polymers. The chemical structure of PDC is illustrated in **Fig. 3**. Because PDC cannot be synthesized from petroleum, attempts have been made to synthesize it from a plant component, lignin, by *Pseudomonas putida* [19]. Recently, microbial production of PDC from glucose was achieved by introducing genes of the shikimate pathway into *Escherichia coli* (*E. coli*) cells. Five genes were introduced to complete the pathway from glucose to PDC through protocatechuate (PCA). In the recombinant *E. coli*, glucose was first converted to protocatechuate (PCA) by 4-hydroxybenzoate hydroxylase (pobA) and dehydroshikimate dehydratase (qutC). PCA

was then converted to PDC by the action of protocatechuate 4,5-dioxygenase (ligA and ligB) and 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (ligC) [20]. The metabolic pathway from glucose to PDC by recombinant *E. coli* is shown in **Fig. 4**. However, it still needs to utilize renewable resource as a raw material for PDC production.

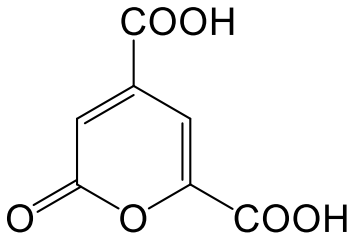

Fig. 3 The chemical structure of 2-pyrone 4,6-dicarboxylic acid (PDC)

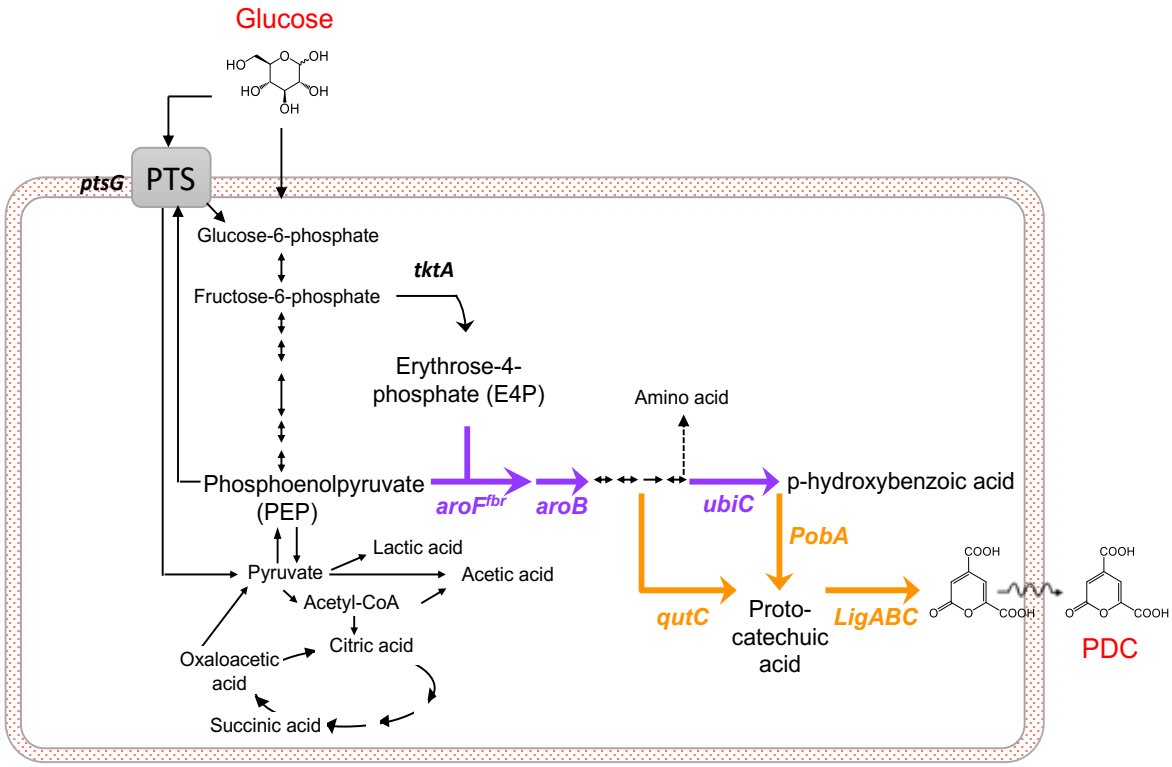

Fig. 4 Metabolic pathway from glucose to PDC [20]

Therefore, in the present study, it was tried to produce PDC using renewable resource. PDC producing recombinant *E. coli* that designed in the previous study [20] was used for the fermentation process and named that *E. coli* stain "original strain". And, the original strain with minor modification was used as the fermentation organism (namely "modified strain"). To establish PDC production process using different biomass feedstocks, the scope of the experimental work is designed as illustrated in **Fig. 5**. The present study aims to extend the process for 2-pyrone 4,6-dicarboxylic acid (PDC) production by using biomass feedstocks. The recombinant *E. coli* pathway from biomass feedstocks to PDC was presented in **Fig. 6**.

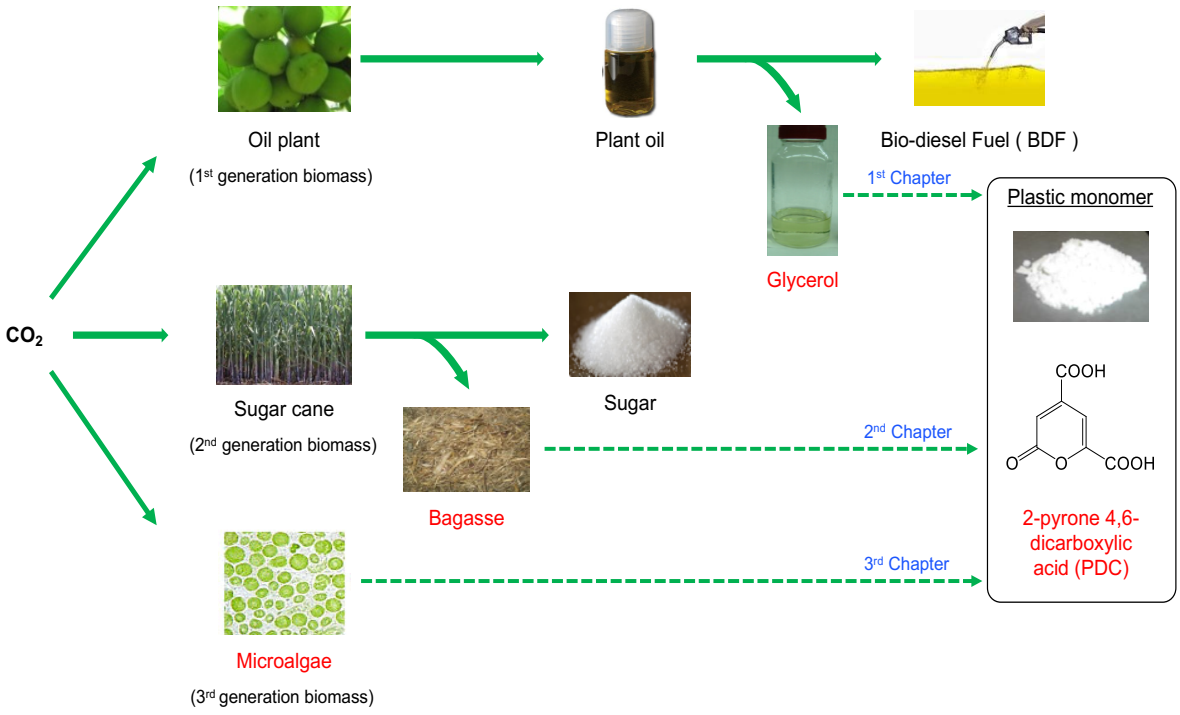

Fig. 5 Outline of the present study for PDC production from different biomass feedstocks

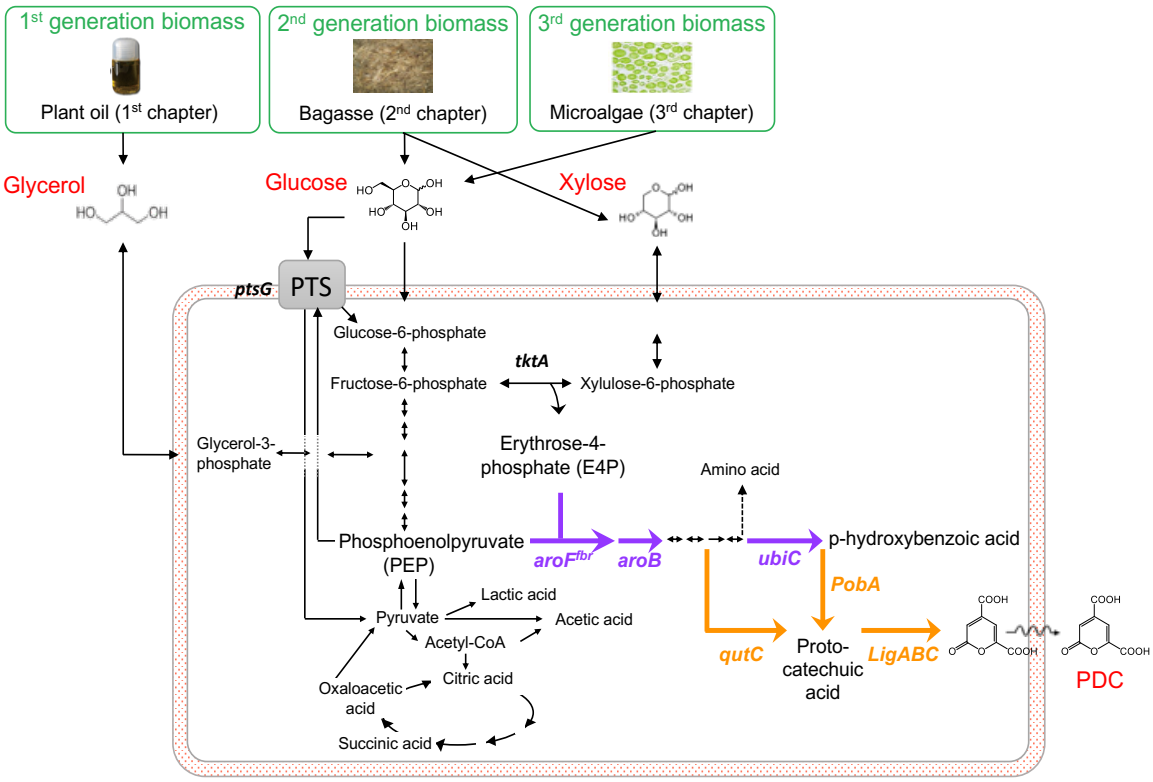

Fig. 6 Metabolic pathway from glycerol, glucose, and xylose to PDC based on [20]

In Chapter 1, the process for PDC production from glycerol, 1,2,3-propanetriol, (by-product of biodiesel) is explained. To investigate the possibility of the fermentative substrate, the enzymatic glycerol (via transesterification process in the presence of enzyme) and the alkali glycerol (via transesterification process in the presence of alkali) were used. The purity of enzymatic glycerol is higher than alkali glycerol. Therefore, the inhibitory effect was monitored by cell growth and PDC production. The original strain and modified strain were applied for fermentation process to examine their effect on PDC production.

Chapter 2 deals with the bagasse hydrolysate for the production of PDC. The first step is the pretreatment of bagasse (by-product from the sugar production) followed by enzymatic hydrolysis and fermentation. Bagasse was pretreated with ionic liquid (choline acetate) and hydrolyzed with cellulase. In the bagasse hydrolysate, glucose and xylose are the main products and the other products are supposed to be amino acid and nucleic acid from the decomposition of cellulose or hemicellulose. Yeast extract was used as the additional nutrient to examine its effect on bagasse hydrolysate. The original strain and modified strain were applied for fermentation process to investigate their effect on PDC production.

Chapter 3 covers the process for PDC production from microalgae. Three green microalgae (*Chlorella emersonii*, *Parachlorella kessleri*, and *Chlorella vulgaris*) were cultivated, starch-accumulated and, analyzed the carbohydrate content. The alga contained high carbohydrate was selected for the preparation of algae hydrolysate. In algae hydrolysate, it contains not only glucose, but proteins, lipids, vitamins, and metals also include based on the composition of algae. To evaluate the effect of the additional nutrient on algae hydrolysate, tryptone and yeast extract were used as the additional nutrient. Macronutrient (total organic carbon and total nitrogen) and micronutrient (Mn^{2+} and Fe^{2+}) in the algae hydrolysate and fermentation media were analyzed. Furthermore, fermentation by the original strain was conducted using algae hydrolysate alone with no additional nutrient, even no use of a basal medium, to obtain high PDC production.

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Chapter 1

PDC Production Process from Glycerol (By-product of Biodiesel)

1.1 Introduction

The utilization of bioresources in place of non-renewable resources is of great interest in the field of industrial polymer production [1]. Fossil resources are limited [2] and cause the emission of greenhouse gases to the atmosphere, which leads to climate change and global warming [3]. To circumvent this problem, alternative feedstocks have been investigated to replace the social system based on petroleum to bioresources. Biodiesel is produced mainly from plant oil, so-called first-generation biomass. The use of plant oil is directly competitive with food and animal feed [4]. It has been considered to produce biodiesel economically. One of the considerations is integrating the process to produce the valuable products using by-product glycerol. Glycerol, 1,2,3-propanetriol, is a by-product of synthesizing biodiesel, oleochemical, and bioethanol from biomass feedstocks. The main contaminants in the crude glycerol are methanol, salts, soaps, and water. Depending on the process conditions and oil source, the concentration and type of contaminant will vary [5-8]. During transesterification process of triglyceride and methanol in the presence of enzyme or alkali, 10 lbs of crude glycerol were obtained as the by-product for every 100 lbs of product biodiesel [8, 9]. Because of its readily available and versatility, glycerol is widely used in industries (eg. cosmetic, pharmaceutical, etc.). The structure of glycerol can be considered as a "mini-sugar" and applied for the conversion of various products such as 1,3-propanediol and succinic acid. Glycerol can convert to fuels or chemicals with high yields compared with carbohydrates because of high reduction of carbon atoms in glycerol [10-13]. Although 2-pyrone 4,6-dicarboxylic acid (PDC) production from pure glucose was successful by original PDC producing *E. coli* (namely “original strain” in the present

study) [14], it would be beneficial to produce from an inexpensive by-product, glycerol. In this chapter, the valuable product, PDC, was produced from the different types of low-priced glycerol. By-product glycerol was obtained from biodiesel process using enzyme or alkali catalysts. These by-products glycerol were called enzymatic glycerol and alkali glycerol in the present study. Fermentation was conducted using original strain and PDC producing *E. coli* strain with minor modification (namely "modified strain" in the present study). The process outline is illustrated in **Fig. 1.1**.

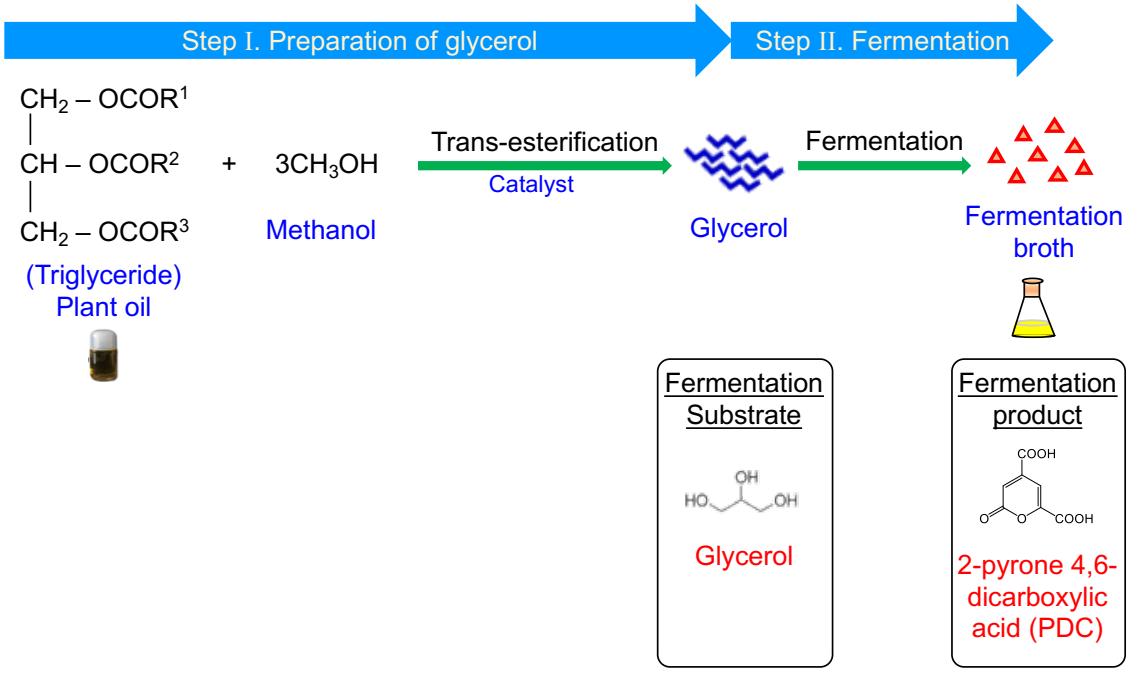

Fig. 1.1 Outline for the production of PDC from glycerol (by-product of biodiesel)

1.2 Experimental

1.2.1 Oil, Bacteria, and plasmids

Waste cooking oil and waste edible oil were collected in local area. Bacterial strain, *E. coli* BL21(DE3) harboring three plasmids (pACYC-aroFfbr-aroB, pCDF-ubiC-pobA, and pFT-LigABC-qutC), original strain, was used as PDC producer [14]. Furthermore, phosphotransferase system (PTS) in recombinant *E. coli* was deleted to terminate some reactions for saving more phosphoenolpyruvate (PEP) molecules to enhance PDC production. This modified strain, also, was used as PDC producer. The other chemicals were reagent grades and commercial source (Nacalai Tesque, Japan).

1.2.2 Enzymatic reaction for biodiesel production

Biodiesel was produced by the reaction of waste cooking oil and methanol in the presence of an enzyme. Biodiesel production method was referred to Hama, et.al (2013) [15]. In a brief, waste cooking oil (1000 g) and 0.5 mol. equivalent of methanol were mixed and supplied to the reaction column at 600 ml/h flow rate. Until the reaction mixture was empty in the tank, the effluent was collected. After that, the effluent mixing with 0.5 mol. equivalent of methanol was supplied again to the previous column. Oleic acid was used to mix with waste cooking oil for preparation of oil with different acid values. Finally, the by-product glycerol was collected from the glycerol separating tank and analyzed the composition.

1.2.3 Alkali reaction for biodiesel production

Waste edible oil (500 g) was put into the flask and heated. Methanol and sodium hydroxide mixture was added to the flask after the temperature was reached to appropriate point. Then, the mixture was stirred continuously (400 rpm). The mixture was transferred to a separating funnel when the temperature reached the predetermined point. It was allowed for overnight in the funnel. The lower layer containing glycerol, methanol, and most parts of catalyst was removed by opening the valve and the upper layer containing biodiesel (fatty acid methyl ester), a small amount of methanol, and catalyst was washed by warm water. Washing was repeated until pH of lower layer was similar to that of washed water. Finally, biodiesel was heated and dried to be free of water. The general scheme for biodiesel process is illustrated in **Fig. 1.2**.

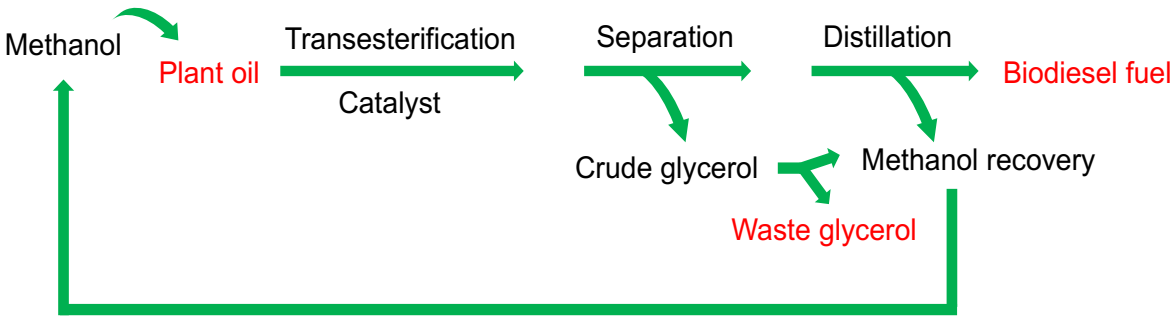

Fig. 1.2 Schematic drawing for the production of biodiesel

1.2.4 Fermentative production of PDC from glycerol

As a fermentation medium, enzymatic or alkali glycerol was diluted with ultrapure water to set the initial concentration of the fermentation media 20 g/L. Fermentation media were supplemented with yeast extract (5 g/L). As a basal medium, M-9 medium (NH_4Cl: 4 g/L, KH_2PO_4: 12 g/L, Na_2HPO_4: 24 g/L, NaCl: 0.5 g/L, $MgSO_4$: 4 mM, Thiamin HCl: 0.04 g/L, $CaCl_2$: 0.4 mM, trace elements solution: 100 L) was used. Trace elements solution was prepared by ($CuSO_4.5H_2O$: 0.079 g/L, $Co(NO_3)_2.6H_2O$: 0.049 g/L, H_3BO_3: 2.86 g/L, $MnCl_2.4H_2O$: 1.81 g/L, $Na_2MoO_4.2H_2O$: 0.39 g/L, $ZnSO_4.7H_2O$: 0.222 g/L). Medium and equipment were sterilized using autoclave (TOMY LSX-700, Tokyo, Japan). For pre-culture, the PDC producing *E. coli* (original strain or modified strain) was cultured overnight at 37 °C, 135 rpm in a test tube containing 5 mL LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L). Pre-cultivation was carried out in shaker incubator (TAITEC, G.BR-200, Tokyo, Japan). Antibiotics, ampicillin (100 mg/L), streptomycin (10 mg/L), and chloramphenicol (30 mg/L), were added to the medium. After pre-culture, the *E. coli* cells were collected by centrifugation (15,000 rpm, 2 min) and the supernatant was discarded. Next, the *E. coli* cells were inoculated into baffled flask containing 50 mL fermentation medium at the initial OD_{600} of 0.1. Throughout the fermentation experiment, three antibiotics described above was added to the fermentation medium. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 M) was added at 0 h of fermentation as an inducer. Fermentation was carried out at 37 °C with constant stirring at 135 rpm. Samples were collected every 3 h until 12 h and then every 12 h until the end of the fermentation at 48 h. Fermentation process for PDC production from glycerol is illustrated in **Fig. 1.3**.

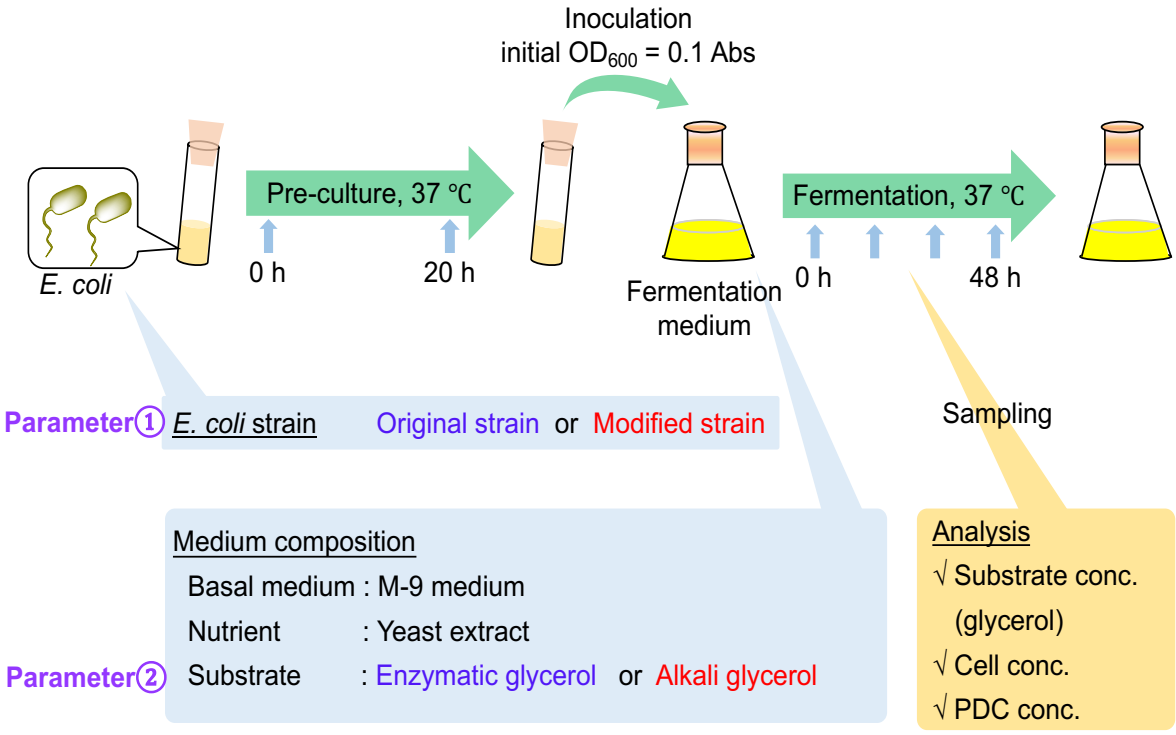

Fig. 1.3 Schematic drawing of experimental conditions for fermentation of glycerol

1.2.5 Analysis for PDC fermentation

The *E. coli* cell growth was monitored by OD_{600} using a DU730 spectrophotometer (Beckman-Coulter), and cell concentration was determined using a coefficient of 0.39 g/L per OD_{600}. Biodiesel, oleic acid, and triglycerides were analyzed by gas chromatography (Shimadzu Co., Kyoto, Japan) with ZB-5HT column. The injector temperature was 320 °C and the detector and column temperatures were 370 °C [15]. The glycerol and PDC concentration in the fermentation media were determined by using high-performance liquid chromatography (HPLC) using a UV/RI detector (SPD-20A / RID-10A, Shimadzu Co., Kyoto, Japan) and ICSep ICE-ION-300 column. The operating conditions were a flow rate of 0.4 mL/min of 0.0085 M H_2SO_4 mobile phase with a column temperature of 70 °C. Total fatty acid analysis was carried out using Gas chromatograph mass spectrometer (GCMS-QP 2010, Shimadzu Co., Kyoto, Japan).

1.3 Results and Discussion

1.3.1 Composition of enzymatic and alkali glycerol

Before the fermentation for PDC production by the original stain or modified strain, the composition of glycerol was analyzed. It was found that glycerol content was 82.3 (wt. %) obtained via transesterification process in the presence of enzyme (see in **Fig. 1.4 A**). The retained components are methanol, fatty acid methyl ester (FAME), water, and other substances [15]. The total fatty acid might contain in the composition of the other substance. For the case of alkali glycerol obtained via transesterification process in the presence of alkali (see in **Fig.1.4 B**), the glycerol content was 56.5 (wt. %) and the remained components are total fatty acid and other substances. Methanol, FAME, and water might contain in the composition of the other substances. The compositions of enzymatic and alkali glycerol are shown in **Fig. 1.5**. The total fatty acid composition of alkali glycerol can be seen in **Fig. 1.6**.

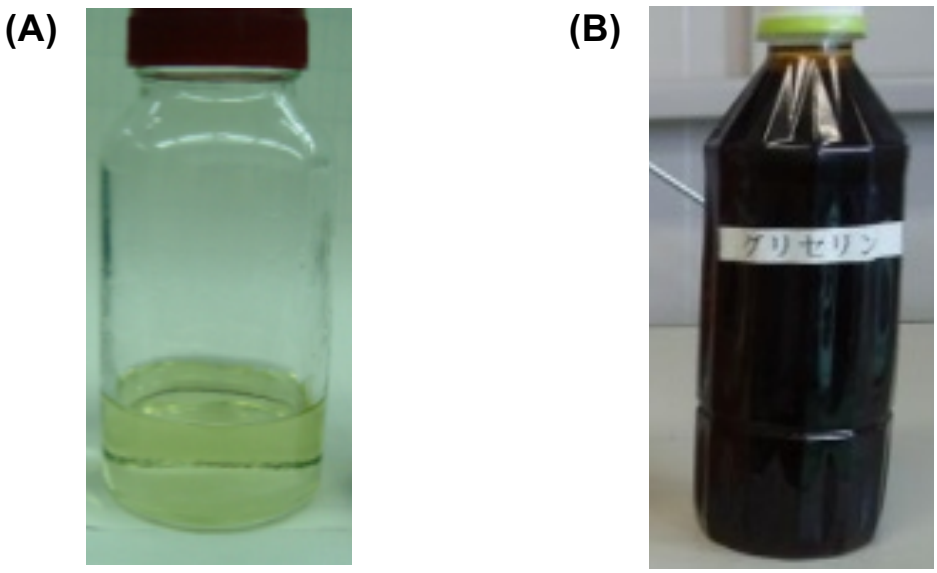

Fig. 1.4 Photograph of (A) enzymatic glycerol and (B) alkali glycerol

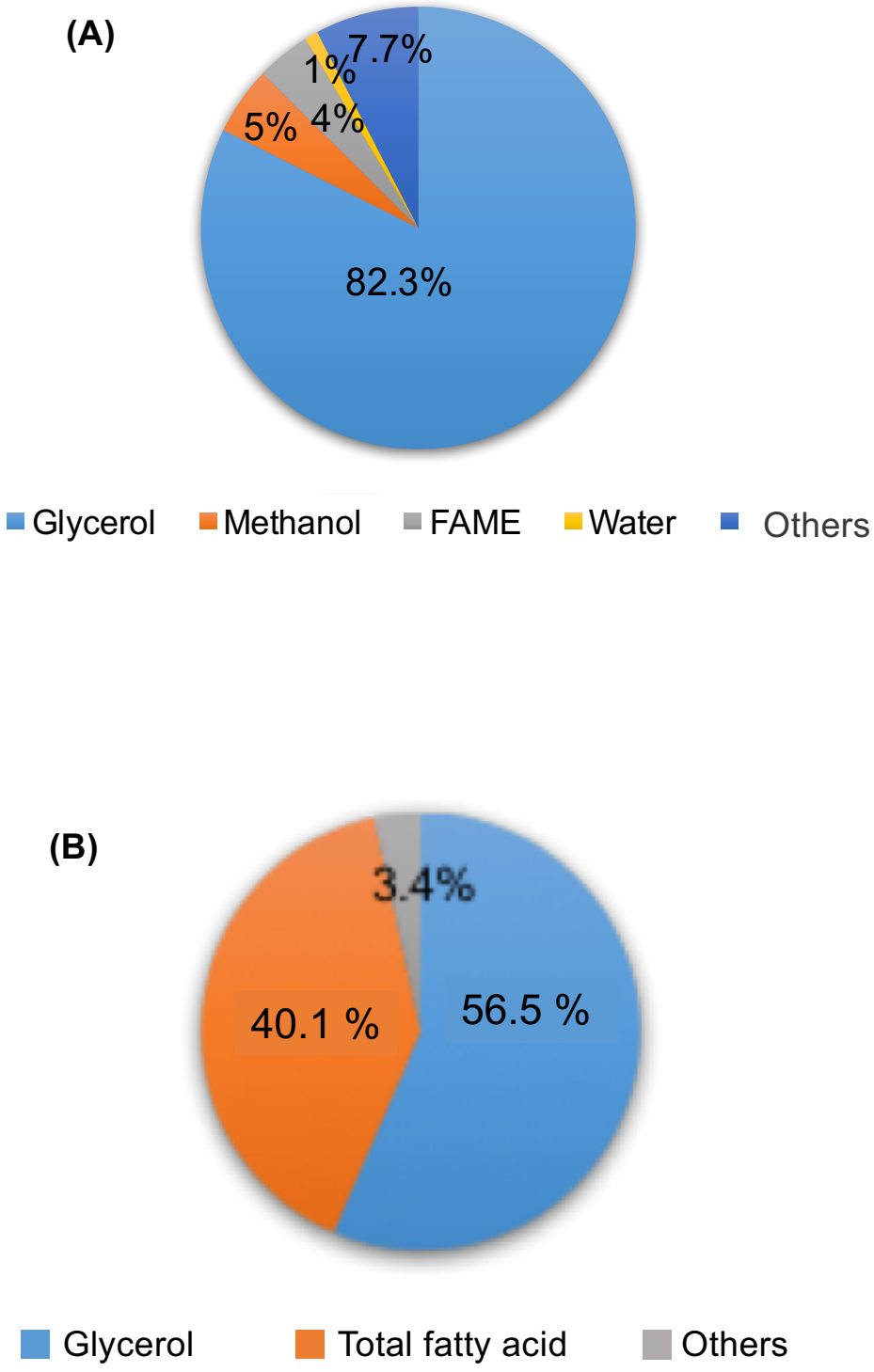

Fig. 1.5 Composition of (A) enzymatic glycerol [15] and (B) alkali glycerol

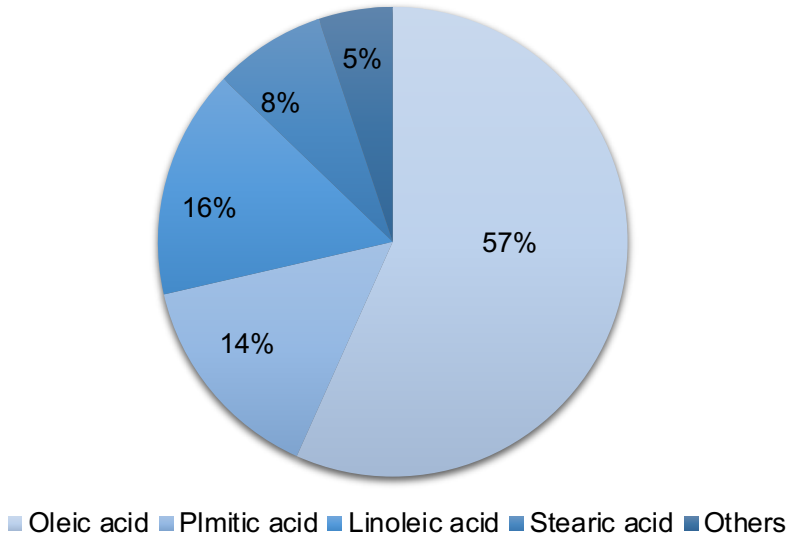

Fig. 1.6 Composition of total fatty acid in alkali glycerol

1.3.2 Effect of glycerol source on PDC production by original strain

Firstly, to examine the availability of glycerol as a substrate for PDC production, the microbial fermentation was carried out using the enzymatic glycerol or alkali glycerol, where the medium was supplemented with yeast extract and the initial glycerol concentration was adjusted to be 20 g/L. Glycerol was consumed with elapsed time, being depleted at 36 h irrespective of the media (see in **Fig. 1.7 A**). As shown in **Fig. 1.7 B**, in the case of enzymatic glycerol, the cell concentration reached a saturated value of 5.6 g/L just after the glycerol depletion at 36 h. The similar condition was observed in the alkali glycerol medium where the saturated cell concentration was 6.8 g/L at 36 h. As shown in **Fig. 1.7 C**, PDC concentration reached 1.9 g/L and 1.6 g/L at 36 h in enzymatic and alkali glycerol media, respectively.

The yield of PDC (mol. %) in enzymatic medium was 4.9 % and for the case of alkali medium, PDC yield was 4.1 %. The similar PDC yield was obtained from different glycerol source. It was considered that the contaminants such as methanol and fatty acid methyl ester did not inhibit the cell growth and PDC production in enzymatic glycerol medium. Cell growth and PDC production also were not inhibited by 40.1 (wt. %) of total fatty acid (the main contaminant) in alkali glycerol medium. Therefore, both type of glycerol can be used as fermentative substrate for PDC production.

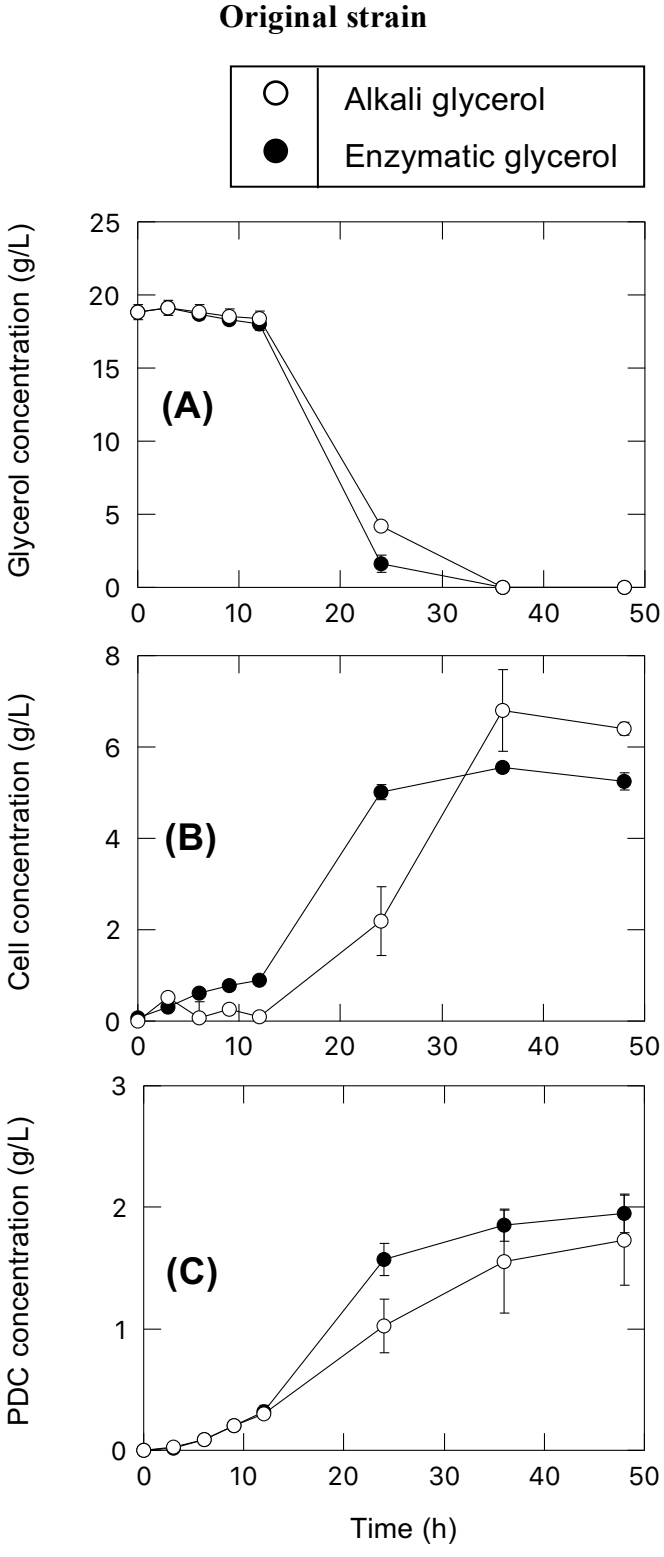

Fig. 1.7 Time courses of (A) glycerol, (B) cell, and (C) PDC concentration during fermentation using enzymatic and alkali glycerol media supplemented with yeast extract. Initial glycerol concentration in the fermentation media was set to be 20 g/L. The error bars indicate the standard deviation from three independent experiments.

1.3.3 PDC production from different glycerol source by modified strain

The microbial fermentation was carried out using the enzymatic glycerol or alkali glycerol, where the medium was supplemented with yeast extract and the initial glycerol concentration was adjusted to be 20 g/L. It was for examining the effect of modified strain on PDC production. As shown in **Fig. 1.8 A**, glycerol was consumed with elapsed time, being depleted at 36 h in enzymatic glycerol medium. But for the case of alkali glycerol medium, glycerol was depleted at 48 h. As shown in **Fig. 1.8 B**, in the case of enzymatic glycerol, the cell concentration reached a saturated value of 6 g/L just after the glycerol depletion at 36 h. In the alkali glycerol medium where the saturated cell concentration was 5.6 g/L at 48 h. As illustrated in **Fig. 1.8 C**, the PDC concentration reached 1.9 g/L at 36 h in enzymatic glycerol medium. On the other hand, in alkali glycerol medium, the PDC concentration reached 1.6 g/L at 36 h.

The yield of PDC (mol. %) in enzymatic glycerol medium was 4.7 % and for alkali glycerol medium, PDC yield was 3.0 %. The yield of PDC was not significantly differenced for each glycerol medium. The slight difference might be due to the purity of enzymatic and alkali glycerol. PDC productivity of by-product glycerol by original strain and modified strain is summarized in **Table 1.1**. The yield of PDC obtained by original strain (4. 9 % for enzymatic and 4.1 % for alkali glycerol) and modified strain (4.7 % for enzymatic and 3.0 % for alkali glycerol) was comparable. It showed that PTS deleting for saving more PEP molecules did not effect for glycerol substrate. It is because glycerol assimilation does not relate to PTS (see in **Fig. 1.9**).

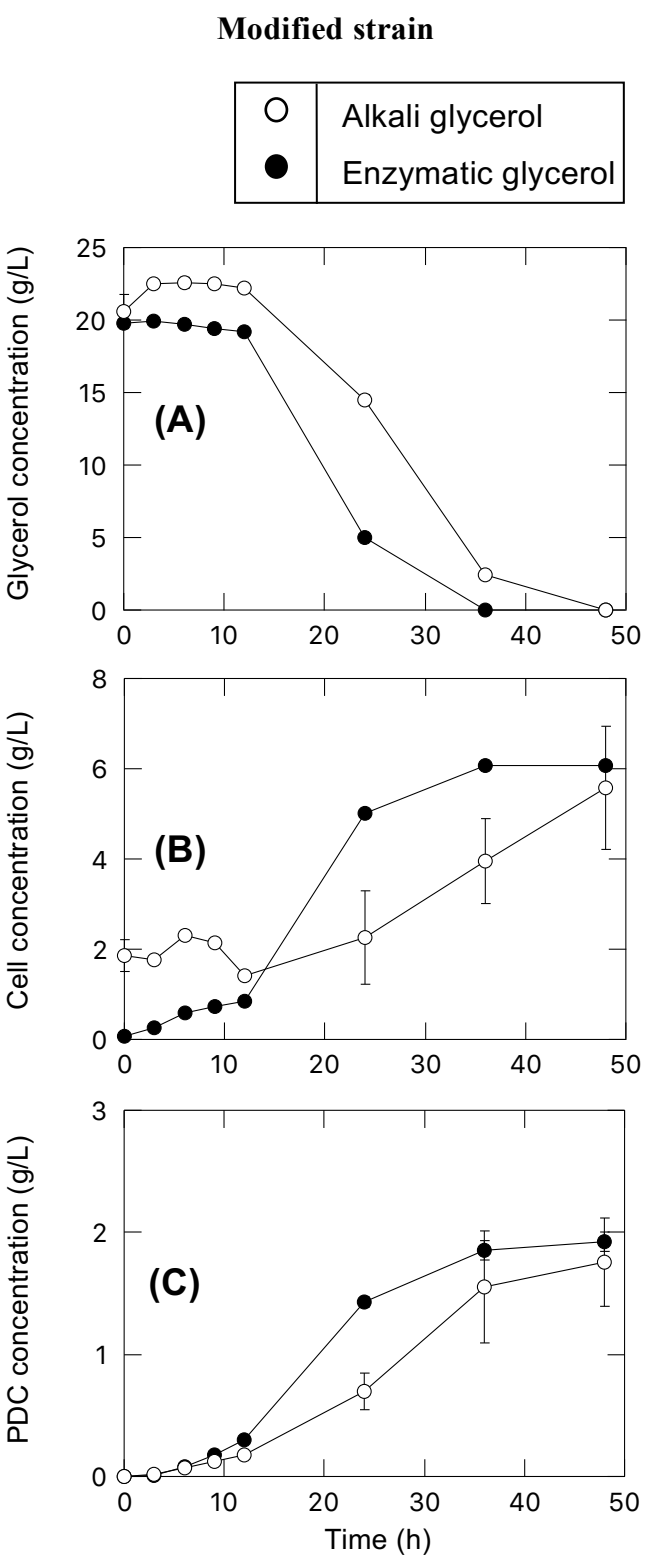

Fig. 1.8 Time courses of (A) glycerol, (B) cell, and (C) PDC concentration during fermentation using enzymatic and alkali glycerol media supplemented with yeast extract. Initial glycerol concentration in the fermentation media was set to be 20 g/L. The error bars indicate the standard deviation from three independent experiments.

Table 1.1 PDC productivity of by-product glycerol

Operational parameter		Basal medium	PDC yield (mol. %)
① Strain	② Substrate		
Original	Enzymatic glycerol	M – 9	4.9
	Alkali glycerol		4.1
Modified	Enzymatic glycerol	M – 9	4.7
	Alkali glycerol		3.0

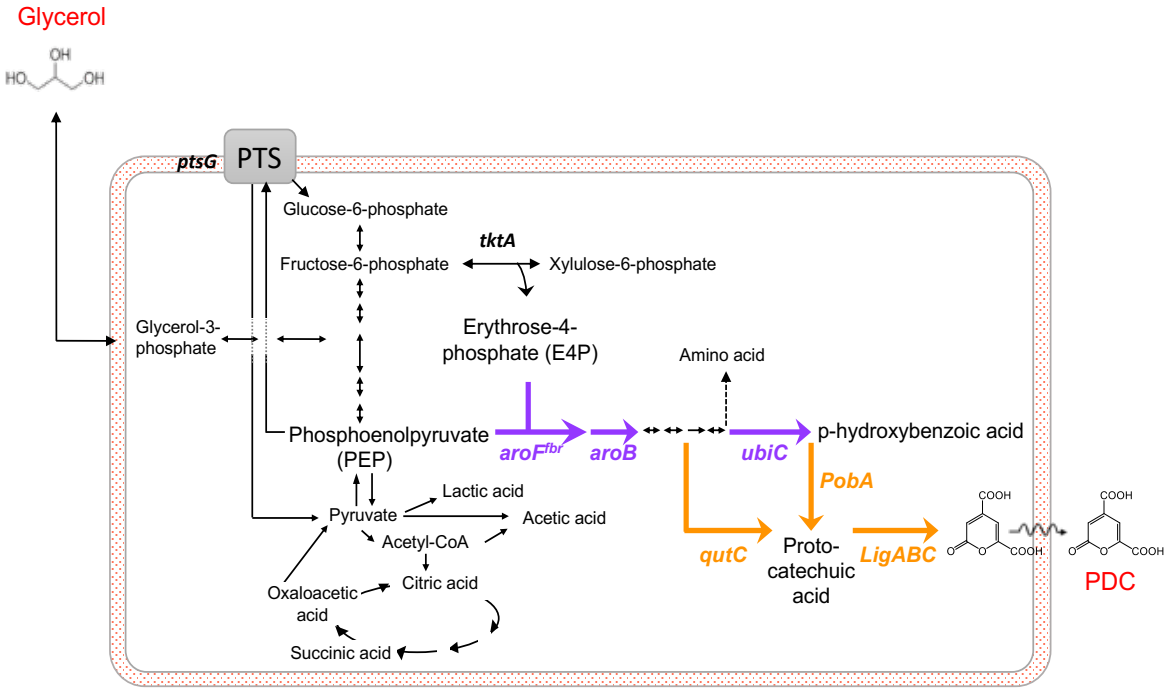

Fig. 1.9 Metabolic pathways from glycerol to PDC

1.4 Summary

The effect of glycerol source and recombinant *E. coli* strains on cell growth and PDC production were examined. M-9 medium was used as the basal medium. It could be concluded as follows.

(1) Glycerol obtained from transesterification process in the presence of enzyme as catalyst has higher purity (82.3 wt. %) than glycerol (56.5 wt.%) from transesterification process in the presence of alkali. The contaminants such as methanol and total fatty acids in enzymatic and alkali glycerol did not inhibit cell growth and PDC production in the media.

(2) The yields of PDC (mol. %) for enzymatic glycerol (4.9 %) and alkali glycerol (4.1 %) by original strain were similar.

(3) Also, the yields of PDC obtained by modified strain are comparable. It was observed that 4.7 % and 3.0 % for enzymatic and alkali glycerol, respectively.

(4) PDC production by original strain and modified strain were not significantly different. It showed that deleting phosphotransferase system (PTS) did not effect on PDC production. It is because glycerol uptake pathway in *E. coli* does not relate to PTS. The slight difference of PDC production between glycerol source was due to the purity of enzymatic and alkali glycerol.

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Chapter 2

PDC Production Process from Bagasse (By-product of Sugar)

2.1 Introduction

Lignocellulosic biomass, so-called second-generation biomass, has been received great attention as alternative feedstocks for fuels and chemicals production. Lignocellulosic material from agricultural residue generates a huge amount [1]. For example, sugarcane bagasse, a waste material after a process of sugar production, left approximately 25 % of sugarcane [2]. Agricultural residues are not only abundant and inexpensive but also do not require to use additional land/energy to produce them [3]. The lignocellulosic feedstock is mainly comprised of cellulose (40-50 %), hemicellulose (25-30 %), lignin (15-20 %), and trace components [4-6]. The crystallinity and rigidity of cellulose and lignin structure must be disrupted before effective utilization of embedded polysaccharides [4]. Pretreatment for disrupting of the lignocellulosic structure is the critical process because it can effect on hydrolysis and downstream fermentation processes. The common process for pretreatment of lignocellulosic biomass is acidic or enzymatic hydrolysis [7]. Recently, ionic liquid (IL), organic salt which melting point is below 100 °C, has been greatly interested in pretreatment. Because it is the excellent solvent, for example, it can dissolve cellulose and raw biomass under mind conditions [8]. The composition of hydrolysate, liquid fraction after hydrolysis, can vary depending on the used pretreatment methods even though the same lignocellulosic materials were used. The main products in hydrolysate are sugar and xylose and low amount of other sugars (e.g. arabinose) may be formed [4]. Also, inhibitors for fermenting microorganisms can occur. Some of the inhibitors are acetic acid (released during pretreatment), furfural (from sugar degradation), syringaldehyde (from lignin degradation), and so on [9-12]. Hydrolysate from lignocellulosic biomass has been

applied for producing fuels and chemicals such as bioethanol, biohydrogen, lactic acid, and vanillin [4, 13, 14]. In this chapter, choline acetate (ChOAc) was for the pretreatment of biomass. It has a less inhibitory effect on cellulase [15]. In the recent years, 2-pyrone 4,6-dicarboxylic acid (PDC) was successfully produced by recombinant *E. coli* (namely "original strain" in the present study) [16]. If PDC can be produced effectively from the hydrolysate of lignocellulosic biomass, it would be beneficial. Therefore, in this chapter, hydrolysate from lignocellulose was used as a fermentative substrate by original strain. Moreover, fermentation was conducted using PDC producing *E. coli* strain with minor modification (namely "modified strain" in the present study). The process outline is illustrated in **Fig 2.1.**

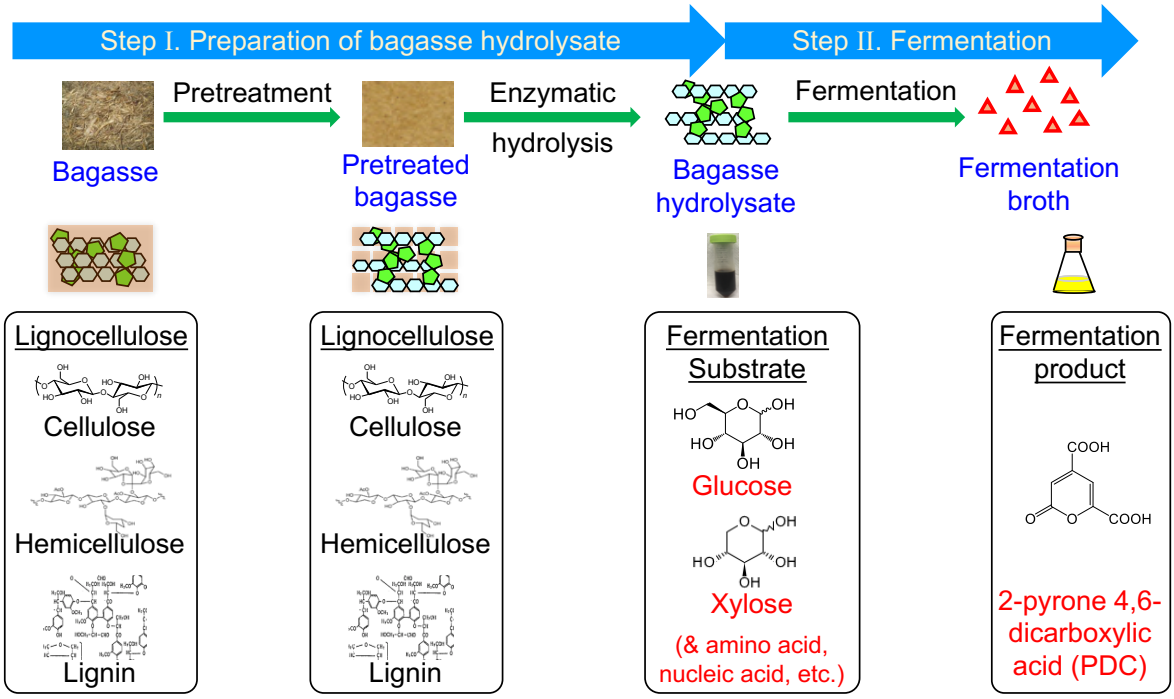

Fig. 2.1 Outline for the production of PDC from bagasse (by-product of sugar)

2.2 Experimental

2.2.1 Bagasse, ionic liquid, cellulase, bacteria, and plasmids

Bagasse powder (approximately 3 mm) was purchased from the Toyota Motor Corporation (Miyoshi, Japan). Ionic liquid, choline acetate (ChOAc), was prepared as in [17]. The cellulase Cellic® CTec2 (batch number VCNI 0008, 106 filter paper units (FPU) per milliliter), was purchased from Novozymes Japan Ltd. (Chiba, Japan). Bacterial strain, *E. coli* BL21(DE3) harboring three plasmids (pACYC-aroFfbr-aroB, pCDF-ubiC-pobA, and pFT-LigABC-qutC), original strain, was used as PDC producer [16]. Moreover, phosphotransferase system (PTS) in recombinant *E. coli* was deleted to terminate some reactions for saving more phosphoenolpyruvate (PEP) molecules to enhance PDC production. This modified strain, also, was used as PDC producer. The other chemicals were reagent grades and commercial source (Nacalai Tesque, Japan).

2.2.2 Pretreatment of bagasse

Bagasse was pulverized to obtain the powder (250-500 m). Pretreatment of bagasse was conducted as described in [17]. Bagasse powder was mixed with 15 g of ionic liquid (IL), choline acetate (ChOAc), and 485 g of water in an open-topped stainless tray. IL solution impregnated bagasse was heated at 110 °C for 5 h to evaporate water. Heating has proceeded for another 16 h. After that, pretreated bagasse was suspended with 500 mL of water and stirred for overnight. And, the suspension was centrifuged (8000 g, 25 °C, 10 min) (KUBOTA 6000, Osaka, Japan) for separating IL and wet pretreated bagasse. The flow chart for pretreatment of bagasse with ionic liquid can be seen in **Fig. 2.2**.

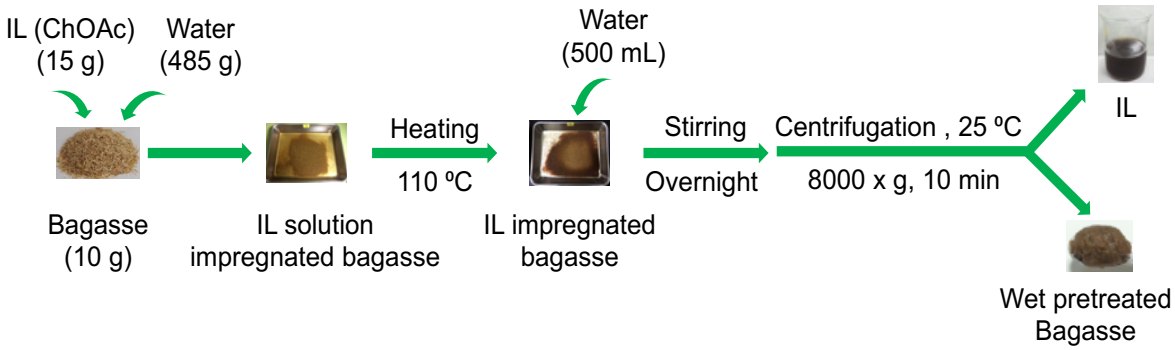

Fig. 2.2 Process for the pretreatment of bagasse [17]

2.2.3 Enzymatic hydrolysis of bagasse

Enzymatic hydrolysis of bagasse was performed as described in [17]. Briefly, wet pretreated bagasse (10 g) was hydrolyzed with an enzyme (CTec2, 100 FPU/g of pretreated bagasse) including water (90 mL). Enzymatic hydrolysis was conducted in 50 mL polypropylene tubes. The tubes were incubated at 50 °C and 35 rpm for 72 h in a heat block (Thermo Block Rotator SN-06BN; Nissinrika Co., Tokyo, Japan). After 72 h hydrolysis, the supernatant was collected by centrifugation (12,000 rpm, 10 min, 25 °C) (KUBOTA 6000, Osaka, Japan). Glucose and xylose content in the collected supernatant was quantified. The flow chart for the enzymatic hydrolysis of pretreated bagasse was illustrated in **Fig. 2.3**.

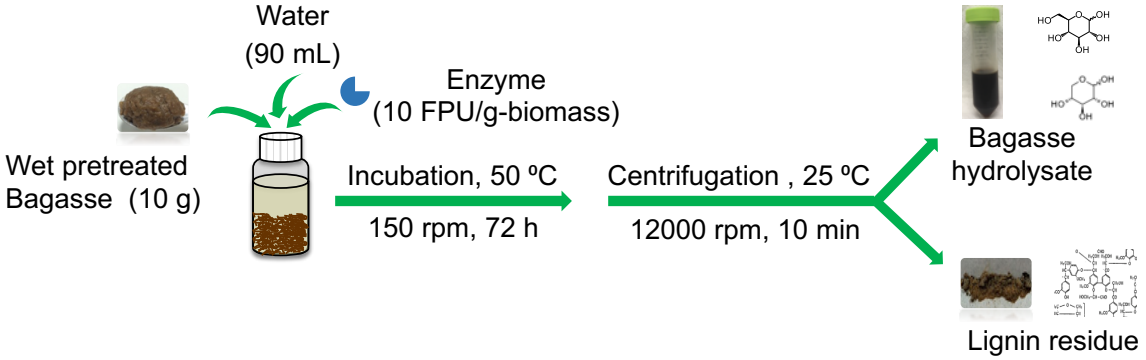

Fig. 2.3 Process for the preparation of bagasse hydrolysate [17]

2.2.4 Fermentative production of PDC from bagasse hydrolysate

As a fermentation medium, the enzyme-hydrolyzed bagasse (bagasse hydrolysate) was diluted with ultrapure water to set the initial concentration of fermentation medium at 20 g/L. This was called as "bagasse hydrolysate medium" in the present study. When necessary, the bagasse hydrolysate medium was supplemented with 2.5 g/L yeast extract as the additional nutrient which contains nitrogen, vitamins, and trace elements [18]. And the concentration of additional nutrient was change to 0 (without yeast extract). As a control medium, “pure sugar (glucose and xylose) mixture medium” was also prepared by replacing the bagasse hydrolysate in the bagasse hydrolysate medium to glucose and xylose. As a basal medium, M-9 medium (NH_4Cl: 4 g/L, KH_2PO_4: 12 g/L, Na_2HPO_4: 24 g/L, NaCl: 0.5 g/L, $MgSO_4$: 4 mM, Thiamin HCl: 0.04 g/L, $CaCl_2$: 0.4 mM, trace elements solution: 100 L) was used. Trace elements solution was prepared by ($CuSO_4.5H_2O$: 0.079 g/L, $Co(NO_3)_2.6H_2O$: 0.049 g/L, H_3BO_3: 2.86 g/L, $MnCl_2.4H_2O$: 1.81 g/L, $Na_2MoO_4.2H_2O$: 0.39 g/L, $ZnSO_4.7H_2O$: 0.222 g/L). Bagasse hydrolysate

medium, pure sugar mixture medium, and trace elements solution were sterilized by filtration (sterile-EO, pore size: 0.2 μm, diameter: 25 mm, Sartorius Stedim Biotech, Germany). The other media and equipment were sterilized using the autoclave (TOMY LSX-700, Tokyo, Japan). To examine the effect of the strain on PDC producing, original PDC producing *E. coli* (original strain) and higher PDC producing *E. coli* (modified strain) were used in this chapter. For pre-culture, the original strain or modified strain was cultured overnight at 37 °C, 135 rpm in a test tube containing 5 mL LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L). Antibiotics, ampicillin (100 mg/L), streptomycin (10 mg/L), and chloramphenicol (30 mg/L), were added to the medium. After pre-culture, the *E. coli* cells were collected by centrifugation (15,000 rpm, 2 min) and the supernatant was discarded. Next, the *E. coli* cells were inoculated into the baffled flask containing 10 mL fermentation medium at the initial OD_{600} of 0.1. Throughout the fermentation experiment, three antibiotics described above was added to the fermentation medium. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 M) was added at 0 h of fermentation as an inducer. Fermentation was carried out at 37 °C with constant stirring at 135 rpm. Samples were collected every 3 h for first 6 h fermentation and then designated point until the end of fermentation at 72 h. Fermentation process for PDC production from bagasse hydrolysate is illustrated in **Fig. 2.4**.

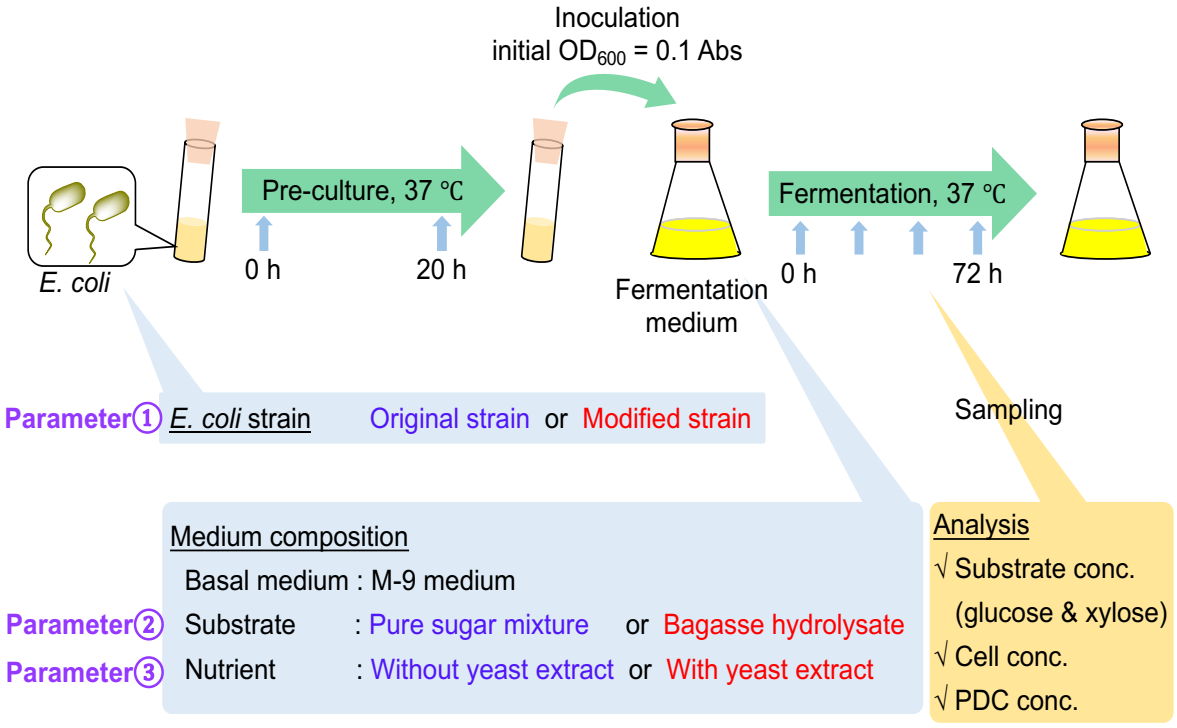

Fig. 2.4 Schematic drawing of experimental conditions for fermentation of bagasse hydrolysate

2.2.5 Analysis for PDC fermentation

The *E. coli* cell growth was monitored by OD_{600} using a DU730 spectro-photometer (Beckman-Coulter), and cell concentration was determined using a coefficient of 0.39 g/L per OD_{600}. Glucose and xylose concentration in the fermentation media were determined by using high-performance liquid chromatography (HPLC) using an RI detector (Shimadzu Co., Kyoto, Japan) and CARBOSep CHO-682 column (Tokyo

Chemical Industry Co., Ltd, Tokyo, Japan). The operating conditions were a flow rate of 0.4 mL/min of water mobile phase with a column temperature of 85 °C. PDC concentration in the fermentation media was determined by using high-performance liquid chromatography (HPLC) using a UV/RI detector (SPD-20A / RID-10A, Shimadzu Co., Kyoto, Japan) and ICSep ICE-ION-300 column. The operating conditions were a flow rate of 0.4 mL/min of 0.0085 M H_2SO_4 mobile phase with a column temperature of 70 °C.

2.3 Results and Discussion

2.3.1 Sugar content in the bagasse hydrolysate

Before the fermentation for PDC production by the recombinant *E. coli*, sugar concentration in the bagasse hydrolysate was evaluated. It was found that glucose concentration was 41.6 g/L and xylose concentration was 16.2 g/L in the bagasse hydrolysate. This bagasse hydrolysate (see in **Fig. 2.5**) was used for PDC production by the recombinant *E. coli*.

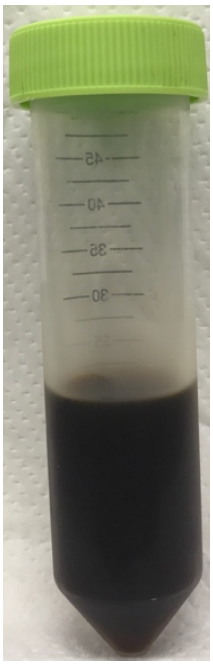

Fig. 2.5 Photo of bagasse hydrolysate

2.3.2 Effect of additional nutrient on cell growth and PDC production by original strain

To evaluate the possibility of bagasse hydrolysate with no additional nutrient as a fermentative substrate for PDC production, the microbial fermentation was conducted with the addition of yeast extract or with no addition of yeast extract. Pure sugar mixture (control) media was prepared the same as in bagasse hydrolysate media preparation. The initial sugar concentration in the media was adjusted to be 20 g/L.

As shown in **Fig. 2.6 A**, the glucose in the pure sugar mixture supplemented with yeast extract was depleted completely at 48 h. For the case of medium without yeast extract, glucose was decreased at 72 h but it was still remained (1.5 g/L) in the medium after fermentation period (72 h). On the contrary, all the glucose was depleted in the bagasse hydrolysate medium containing yeast extract or no yeast extract (see in **Fig. 2.6 B**). Similar phenomena were occurred in xylose concentration. As shown in **Fig. 2.6 C** and **D**, xylose was not decreased in pure sugar mixture without yeast extract medium. As described in **Fig. 2.6 E**, cell concentration reached a saturated value of 3.7 g/L at 48 h in the case of pure sugar mixture with yeast extract; on the other hand, saturated cell concentration was 5.9 g/L at 48 h in bagasse hydrolysate with yeast extract (**Fig. 2.6 F**). Cell concentration 0.4 g/L was confirmed at 48 h in pure sugar mixture without yeast extract medium (**Fig. 2.6 E**). On the contrary, as shown in **Fig. 2.6 F**, the saturated cell growth (5.1 g/L) was obtained from bagasse hydrolysate without yeast extract medium at 48 h. As shown in **Fig. 2.6 G**, PDC concentration reached 4.6 g/L at 48 h in the pure sugar mixture with yeast extract medium. It was comparable to that in bagasse hydrolysate with yeast extract medium. On the other hand, PDC concentration was 0.2 g/L at 48 h in the pure sugar mixture without yeast extract medium but for the case of

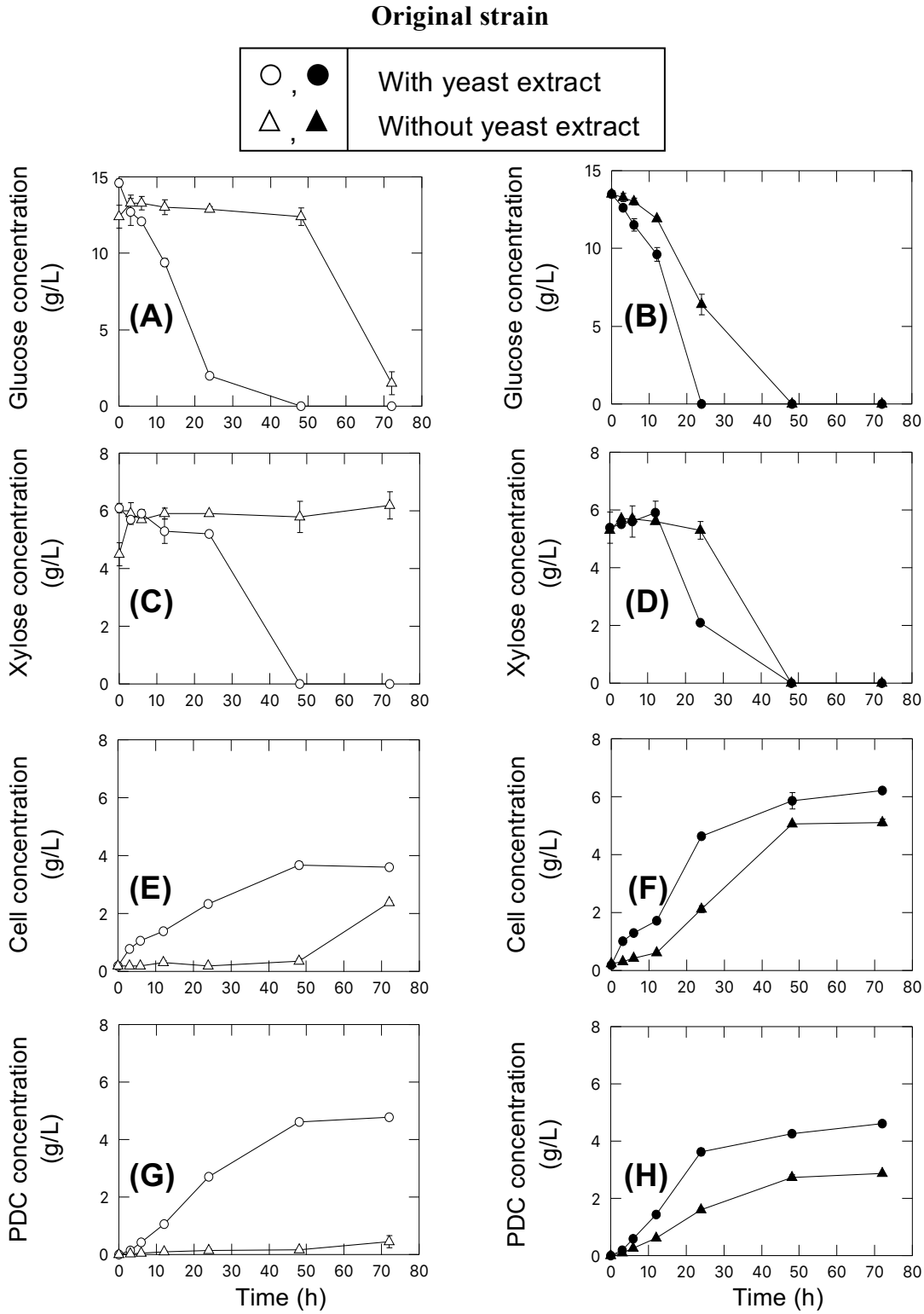

Fig. 2.6 Time courses of (A and B) glucose, (C and D) xylose, (E and F) cell, and (G and H) PDC concentration during fermentation using pure sugar mixture and bagasse hydrolysate media. (A, C, E, and G) Pure sugar mixture medium and (B, D, F, and H) bagasse hydrolysate medium. Initial sugar concentration in the fermentation media was set to be 20 g/L. The error bars indicate the standard deviation from three independent experiments

bagasse hydrolysate without yeast extract medium, PDC concentration reached 2.7 g/L at 48 h (**Fig. 2.6 G and H**).

PDC yield (mol. %) of pure sugar mixture with yeast extract medium was 31.1 % and that of bagasse hydrolysate with yeast medium was 30.9 %. PDC yield was comparable as in PDC concentration for both media. It was supposed that in bagasse hydrolysate medium, it contains other nutrients (e.g. amino acid, nucleic acid, etc.) from the decomposition of cellulose or hemicellulose other than glucose and xylose. The nutrients might be excessive when the bagasse hydrolysate was supplemented with yeast extract. Therefore, it can be considered that PDC yield from bagasse hydrolysate was comparable to pure sugar mixture when media supplemented with yeast extract. PDC yield (mol. %) of pure sugar mixture without yeast extract medium was 1.1 %; on the other hand, 19.7 % was obtained from bagasse hydrolysate without yeast extract medium. These results showed that bagasse hydrolysate without any additional nutrient could be applied as a fermentation medium. It was supposed that amino acid and nucleic acid from the decomposition of cellulose or hemicellulose in bagasse hydrolysate could support as carbon and nitrogen sources for cell growth and PDC production.

2.3.3 Effect of modified strain on cell growth and PDC production

To evaluate the effect of the modified strain on cell growth and PDC production, the microbial fermentation was conducted using bagasse hydrolysate or pure sugar mixture (control) as the fermentative substrate with the addition of yeast extract or with no addition of yeast extract. The initial sugar concentration in the media was adjusted to be 20 g/L.

As shown in **Fig. 2.7 A**, glucose was depleted within 48 h for the pure sugar mixture with yeast extract medium but for the without yeast extract medium, only small

amount of glucose was depleted within fermentation period. For the case of bagasse hydrolysate, glucose and xylose were depleted in the medium with yeast extract or without yeast extract at 48 h (**Fig. 2.7 B and D**). For the case of pure sugar mixture medium (**Fig. 2.7 C**), although xylose was depleted in the addition of yeast extract, only the small amount of xylose was decreased in the addition of no yeast extract. As shown in **Fig. 2.7 E**, cell concentration reached to 3.7 g/L at 48 h in the pure sugar mixture with yeast extract medium; on the contract, cell growth was confirmed as 0.2 g/L at 48 h in the without yeast extract medium. The cell growth profile was similar as in **Fig. 2.6 E**. As shown in **Fig. 2.7 F**, the saturated cell concentrations were 5.4 g/L and 4.8 g/L for bagasse hydrolysate whit yeast extract medium and without yeast extract medium, respectively.

PDC concentration of pure glucose medium with yeast extract reached to 3.2 g/L at 48 h and that of medium without yeast extract was 0.1 g/L (**Fig. 2.7 G**). For bagasse hydrolysate with yeast extract medium, PDC concentration was 5.4 g/L and for the case of without yeast extract medium, PDC concentration reached to 4.8 g/L (**Fig. 2.7 H**). PDC yield (mol. %) of pure sugar mixture with yeast extract medium was 21.9 %. This value was lower than PDC productivity (31.1 %) by the original strain. PDC productivity of by-product bagasse by original strain and modified strain is presented in **Table 2.1**. For the pure sugar mixture without yeast extract medium, PDC productivity was similar for original strain and modified strain. On the contrary, for the case of bagasse hydrolysate with yeast extract medium, PDC yield was 38.1 %. It was higher than PDC productivity (30.9 %) by the original strain under the same medium. Furthermore, PDC yield from bagasse hydrolysate without yeast extract medium was 31.7 %. Also, it was higher than PDC productivity (19.7 %) by the original strain under the same medium. PDC yield (31.7 %) by modified strain using bagasse hydrolysate without yeast extract medium was

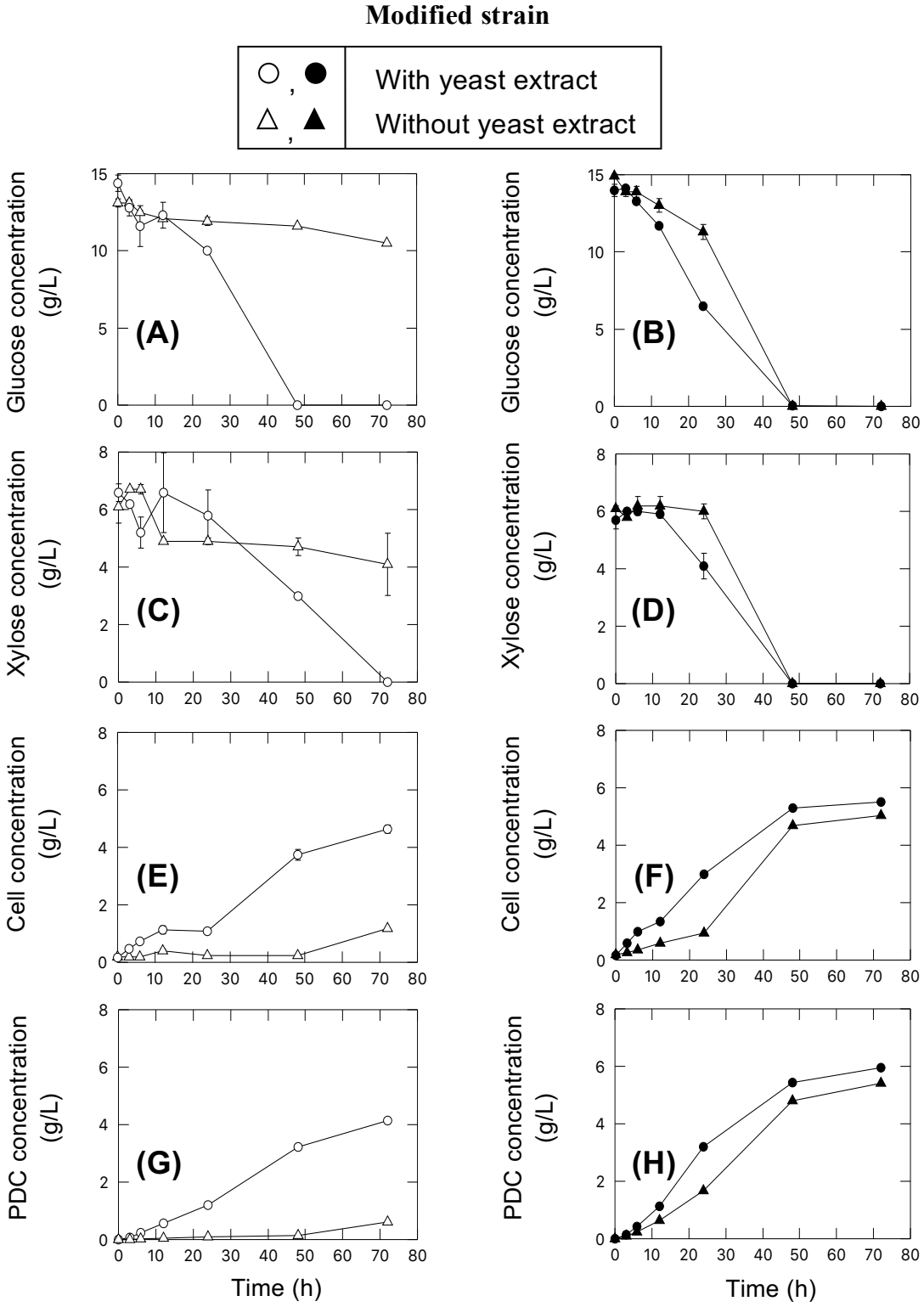

Fig. 2.7 Time courses of (A and B) glucose, (C and D) xylose, (E and F) cell, and (G and H) PDC concentration during fermentation using pure sugar mixture and bagasse hydrolysate media. (A, C, E and G) Pure sugar mixture medium, and (B, D, F and H) bagasse hydrolysate medium. Initial sugar concentration in the fermentation media was set to be 20 g/L. The error bars indicate the standard deviation from three independent experiments

higher than PDC productivity of original strain using bagasse hydrolysate with yeast extract medium. The results showed that PTS deleting in *E. coli* for saving more PEP molecules for PDC production has a significant effect on bagasse hydrolysate substrate. It is because glucose assimilation pathway in *E. coli* relates to PTS. It can be seen in **Fig. 2.8**.

Table 2.1 PDC productivity of by-product bagasse

Operational parameter			Basal medium	PDC yield (mol. %)
① Strain	② Substrate	③ Nutrient		
Original	Pure sugar mixture	With yeast extract	M – 9	31.1
	Bagasse hydrolysate			30.9
	Pure sugar mixture	Without yeast extract	M – 9	1.1
	Bagasse hydrolysate			19.7
Modified	Pure sugar mixture	With yeast extract	M – 9	21.9
	Bagasse hydrolysate			38.1
	Pure sugar mixture	Without yeast extract	M – 9	1.0
	Bagasse hydrolysate			31.7

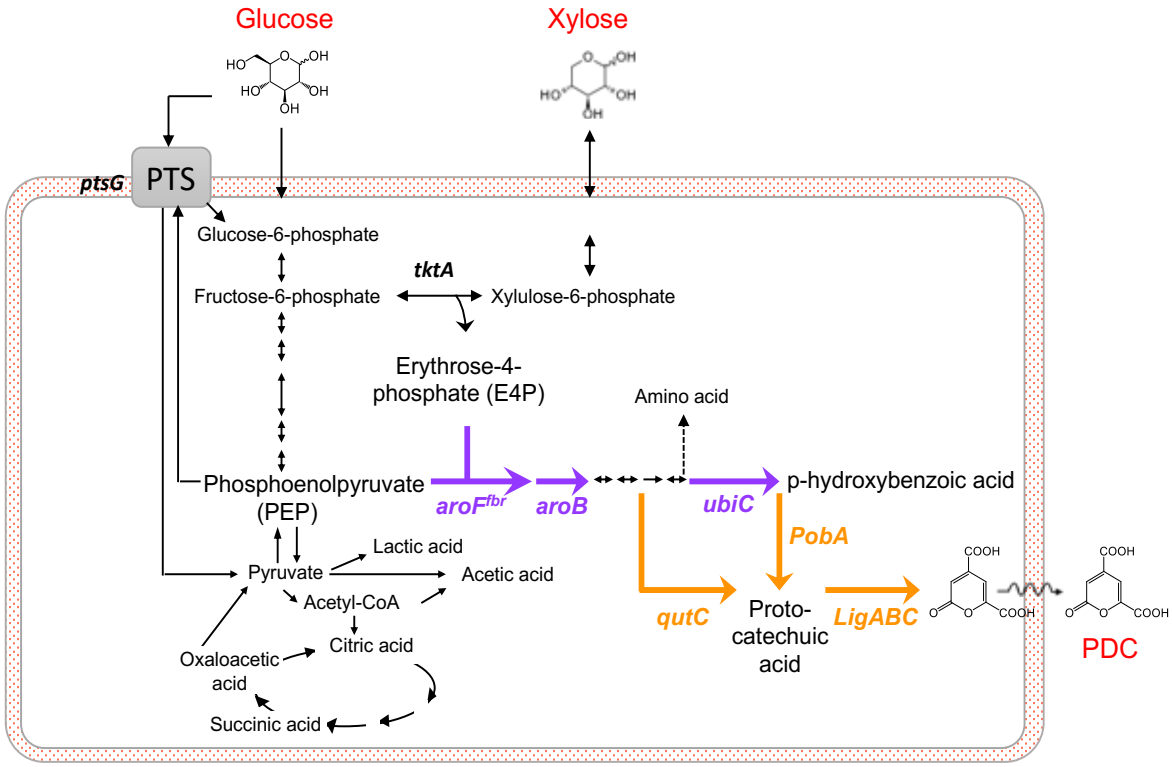

Fig. 2.8 Metabolic pathways from glucose and xylose to PDC

2.4 Summary

The effect of additional nutrient and recombinant *E. coli* strains on cell growth and PDC production using bagasse hydrolysate as the fermentative substrate was studied. M-9 medium was used as the basal medium. The following conclusions could be drawn.

(1) The bagasse hydrolysate contained glucose 41.6 g/L and xylose 16.2 g/L. It was supposed that amino acid, nucleic acid, etc. (from the decomposition of cellulose or hemicellulose) might contain in the bagasse hydrolysate.

(2) The yields of PDC (mol. %) for bagasse hydrolysate with yeast extract medium and without yeast extract medium were 30.9 % and 19.7 % by original strain, respectively. It can be considered that bagasse hydrolysate can produce PDC without using additional nutrient. Amino acid and nucleic from the decomposition of cellulose or hemicellulose in bagasse hydrolysate could be the carbon and nitrogen sources for cell growth and PDC production.

(3) The yield of PDC (mol. %) for bagasse hydrolysate with yeast extract medium by the modified strain was 38.1 %. The yield of PDC (31.7 mol. %) was obtained from bagasse hydrolysate without yeast extract medium. It can be seen that PDC productivity was enhanced by the modified strain for bagasse hydrolysate containing with yeast extract and without yeast extract media. It showed PTS deleting in *E. coli* effects on PDC production.

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Chapter 3

PDC Production Process from Microalgae

3.1 Introduction

Microalgae, so-called third-generation biomass, are considered as one of the alternative biomasses for producing bio-based fuels and chemicals. An advantage of using microalgae as a feedstock is that they grow with only light and carbon dioxide and without the need for an extraneous carbon source [1-4]. Microalgae contain starch and lipids as the main energy reserves under nutrient starvation and variation in growth conditions [5-8]. As a feedstock, microalgae contain no lignin, which simplifies the preparation of fermentable sugars without any complicated pretreatment compared with lignocellulosic biomass. Taking advantage of this, microbial production of fuels and chemicals, such as ethanol and succinic acid fermentation by bacteria and yeast, has been conducted using microalgae as a feedstock [9-11]. Hydrolyzed microalgae have another possible advantage in that they can be used as bacterial medium without adding any extraneous nutrients. Because bacterial species usually require various nutrients, such as a nitrogen source or trace elements, for growth, it is necessary to add extra nutrients in the medium besides a carbon source. From the view point of exogenous elements, the hydrolyzed microalgae alone could contain sufficient nutrients and trace elements for bacterial growth. *Chlorella* species are one candidate because they contain a large amount of carbohydrates (starch and cellulose), lipids, and other nutrients [12]. For example, *C. zofingiensis* can accumulate up to 45 % – 60 % of dry weight of starch and lipid, respectively, under appropriate culture conditions [13]. However, the capabilities of *Chlorella* as a feedstock for growing microbes and producing green-chemicals are yet to be investigated.

Although PDC production from pure glucose was successful [14], it would be ideal to produce the chemical from an actual and cost-effective biomass. In the present study, to evaluate the potential of algae hydrolysate as a nutrient source for PDC fermentation, starch-accumulated microalgae were acid-hydrolyzed and the hydrolysate was then applied for PDC production by original PDC producing *E. coli* (original strain). The process outline is illustrated in **Fig. 3.1**.

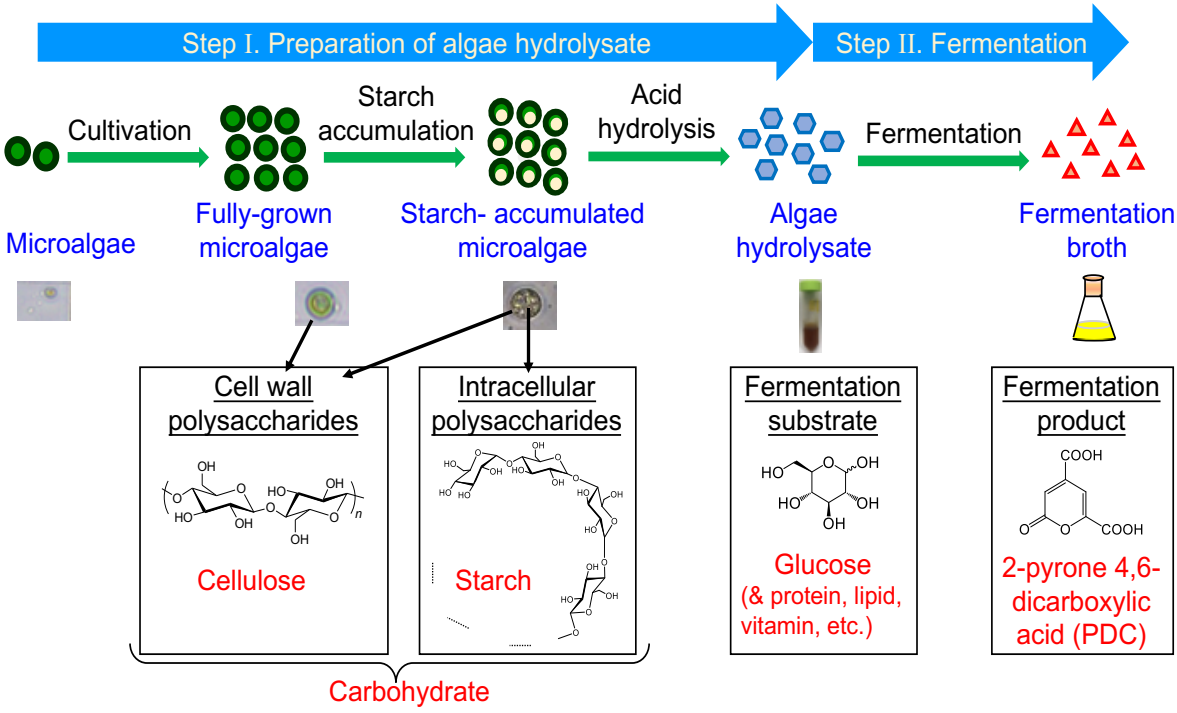

Fig. 3.1 Outline for the production of PDC from algae hydrolysate

3.2 Experimental

3.2.1 Microalgae, bacteria, and plasmids

A pure strain of the green microalgae *Chlorella. emersonii* (NIES-2151), *Parachlorella kessleri* (NIES-2159), and *Chlorella vulgaris* (NIES-2170) were purchased from the National Institute for Environmental Studies, Tsukuba, Japan. Bacterial strain, *E. coli* BL21(DE3) harboring three plasmids (pACYC-aroFfbr-aroB, pCDF-ubiC-pobA, and pFT-LigABC-qutC) was used as PDC producer (namely "original strain" in the present study) [14].

3.2.2 Cultivation of microalgae

Frozen glycerol stock of each microalgae (1 mL) was thawed at room temperature and subsequently pre-cultivated in each 500 mL Erlenmeyer flask containing 250 mL of tris-acetate phosphate (TAP) medium (NH_4Cl: 400 mg/L, $CaCl_2 \cdot 2H_2O$: 51 mg/L, $MgSO_4 \cdot 7H_2O$: 100 mg/L, K_2HPO_4: 119 mg/L, KH_2PO_4: 60.3 mg/L, Hutner's trace elements: 1 mL/L, acetic acid: 1 mL/L, tris (hydroxymethyl) aminomethane: 2420 mg/L), according to a previous report [15]. The medium was sterilized by autoclave (TOMY LSX-700, Tokyo, Japan). The pre-culture in TAP medium was carried out statically (Incubator, EYELA FLI-301N, Tokyo, Japan) at 25 °C under a continuous fluorescent white light (7338 Lux) without aeration.

Next, 10 mL of each pre-cultivated microalgae was transferred to a 2 L glass bottle containing 2 L of TAP medium for proliferation of microalgae cell. When the optical density of 540 nm (OD_{540}) reached approximately 2, the algae culture broth was centrifuged (5,000 rpm, 10 min) (KUBOTA 6000, Tokyo, Japan) and washed with 0.85 % NaCl three times. Subsequently, the cells were transferred to the 2 L glass bottle

containing 2 L of TAP medium without sulfur (S-TAP medium) for starch accumulation in the microalgae cells [16]. Both the cultures in TAP and S-TAP medium were carried out at 25 °C under continuous fluorescent white light and constant mixing with aeration.

During the culturing in the TAP medium, cell growth was monitored by OD_{540} using a spectrophotometer (DU 730, Beckman Coulter, Inc.). During the culturing of starch accumulation in the S-TAP medium, cell concentration was determined by manual counting using hemocytometer (Bacteria counter A161, Sunlead Glass, Japan). Microalgae cultivation process is shown in **Fig. 3.2**.

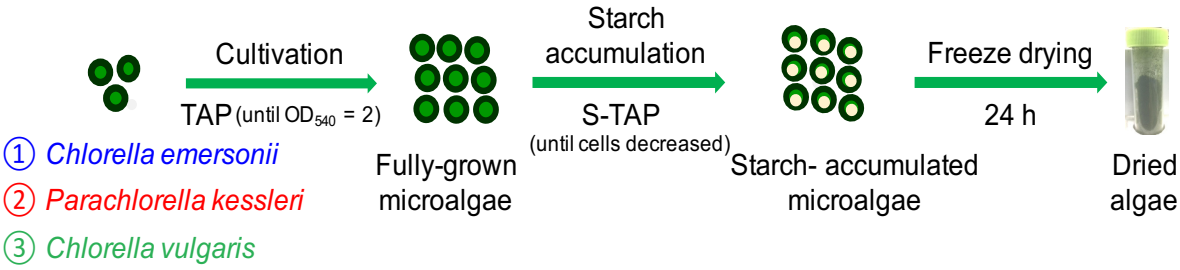

Fig. 3.2 Process for microalgae cultivation

3.2.3 Acid hydrolysis of microalgae

When the microalgae cell entered a stationary phase during culturing in S-TAP medium, the cells were harvested by centrifugation (5,000 rpm, 10 min). After discarding the supernatant, they were freeze-dried and stored at −25 °C in the dark. Then microalgae were freeze-dried (EYELA, FDU-2200, Tokyo, Japan). The freeze-dried microalgae were hydrolyzed using sulfuric acid, based on the National Renewable Energy Laboratory

(NREL/TP-5100-60957) method [17]. Briefly, 25 mg microalgae (dry biomass) was mixed with 250 µL of 72 (wt.%) sulfuric acid. Next, the mixture was incubated (EYELA NTS-4000, Tokyo, Japan) at 30 °C for 1 h. After incubation, the mixture was diluted with 7 mL of water and autoclaved (TOMY, LSX-700, Tokyo, Japan) at 121°C for 1 h. Hydrolyzed samples were neutralized with calcium carbonate and filtered (sterile-EO, pore size: 0.2 µm, diameter: 25 mm, Sartorius Stedim Biotech, Germany). **Figure 3.3** shows the process for acid hydrolysis of microalgae.

To obtain algae-hydrolysate containing high glucose concentration, dried microalgae 500 mg was used instead of 25 mg. The glucose concentration in the hydrolysate was analyzed by the glucose oxidase (GOD) method as described below.

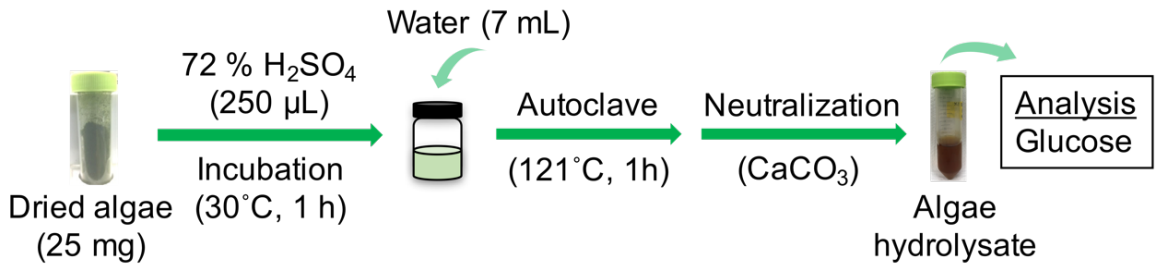

Fig. 3.3 Process for preparation of algae hydrolysis

3.2.4 Fermentative production of PDC from algae hydrolysate

As a fermentation medium, the acid-hydrolyzed microalgae (algae hydrolysate) was diluted with ultrapure water to set the initial concentration of the fermentation media at 2, 5, 10, or 16 g/L. This was called as “algae hydrolysate medium” in the present study.

When necessary, the algae hydrolysate medium was supplemented with 10 g/L tryptone and 5 g/L yeast extract as additional nutrients which contains nitrogen, vitamins, and trace elements [18]. The concentration of additional nutrients of "10 g/L tryptone and 5 g/L yeast extract" was defined as "×1 nutrient" in the present study, and the concentration of additional nutrients was changed to ×1/2, ×1/4, ×1/8, and ×0 nutrient (no additional nutrients). As a control medium, "pure glucose medium" was also prepared by replacing the algae hydrolysate in the algal hydrolysate medium to glucose. Algae hydrolysate and pure glucose media were sterilized by filtration. The other media were sterilized by autoclave.

For pre-culture, the PDC -producing *E. coli* was cultured (Bioshaker, TAITEC, G.BR-200, Tokyo, Japan) overnight at 37 °C, 135 rpm in a test tube containing 5 mL LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L). Antibiotics, ampicillin (100 mg/L), streptomycin (10 mg/L), and chloramphenicol (30 mg/L), were added to the medium. After pre-culture, the *E. coli* cells were collected by centrifugation (15,000 rpm, 2 min) and the supernatant was discarded. Next, the *E. coli* cells were inoculated into Sakaguchi flask containing 50 mL fermentation medium at the initial OD_{600} of 0.1. Inoculation was carried out using bioshaker (G.BR-200, Tokyo, Japan). Throughout the fermentation experiment, three antibiotics described above was added to the fermentation medium, and calcium carbonate (25 g/L) was also added to the fermentation medium to control pH during fermentation. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 M) was added at 0 h of fermentation as an inducer. Fermentation was carried out at 37 °C with constant stirring at 135 rpm. Samples were collected every 3 h until 12 h and then every 12 h until the end of the fermentation at 48 h. Fermentation process for PDC production from algae hydrolysate is described in **Fig. 3.4** and **3.5**.

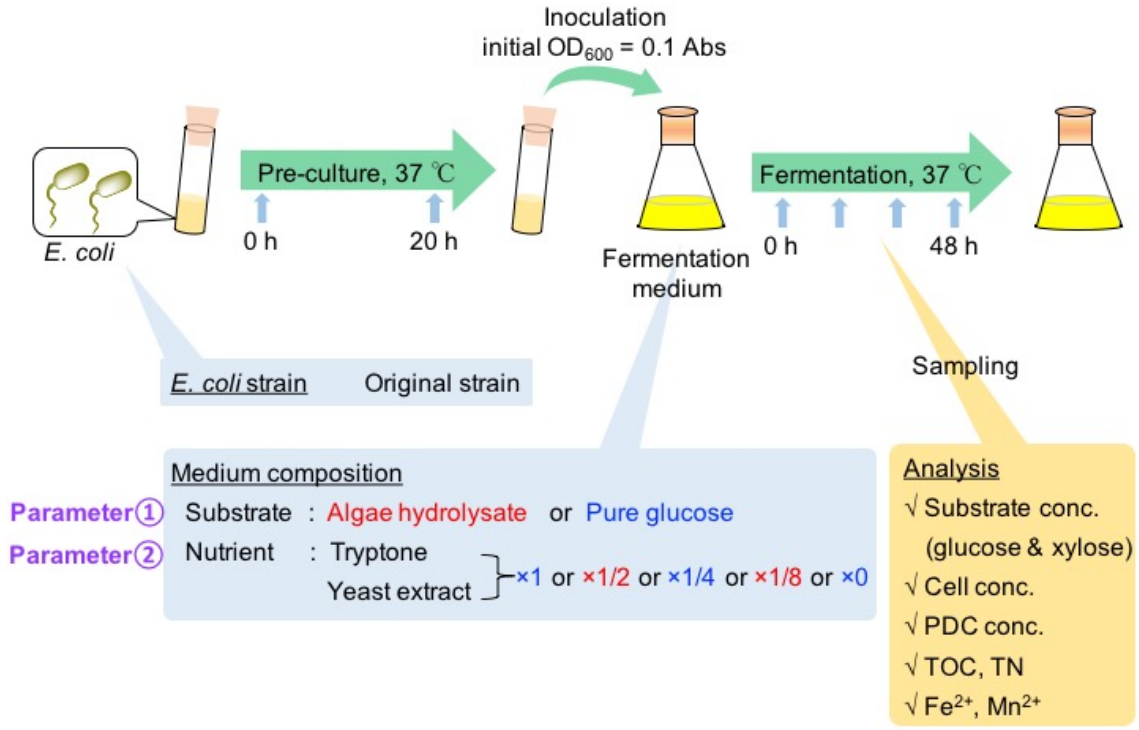

Fig. 3.4 Schematic drawing of experimental conditions for fermentation

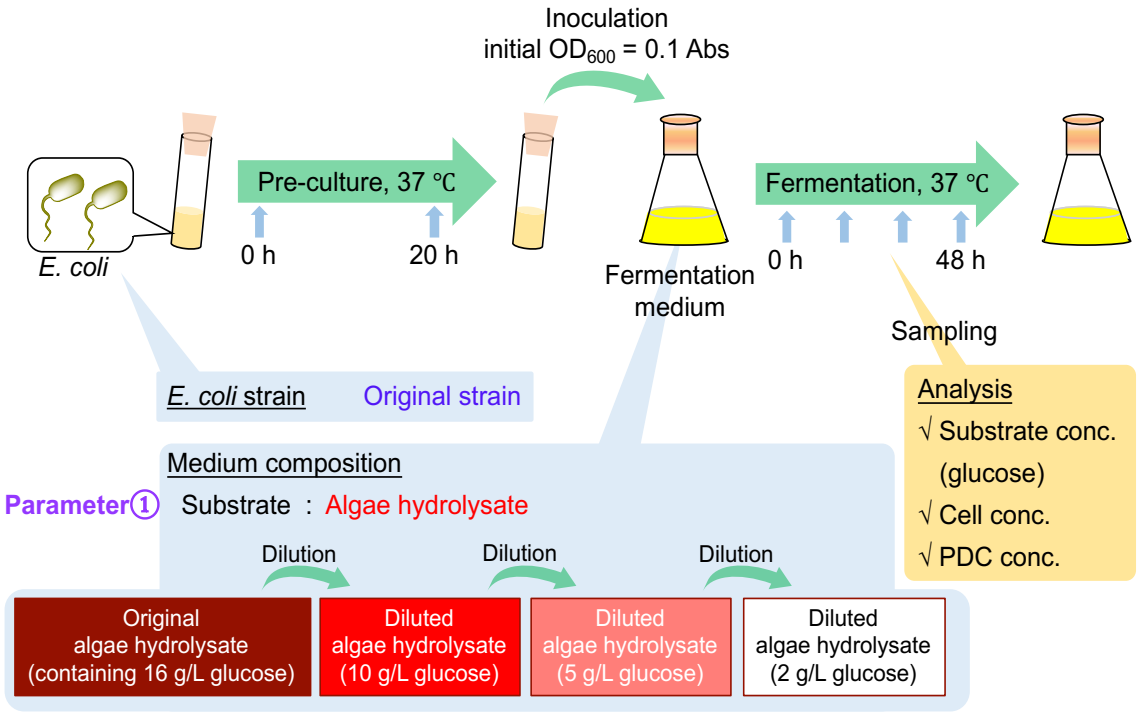

Fig. 3.5 Schematic drawing of experimental conditions for high PDC production

3.2.5 Analysis for PDC fermentation

The glucose concentration in the fermentation medium was determined by the glucose oxidase (GOD) method using the LabAssay Glucose, Mutarotase-GOD method (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Total organic carbon (TOC) and total nitrogen (TN) in the fermentation medium were determined by using a total organic carbon analyzer (TOC-V CPN, Shimadzu Co., Kyoto, Japan). The microelement (Fe^{2+} and Mn^{2+}) concentration in the fermentation medium was determined by using atomic absorption spectrophotometry (AA-7000, Shimadzu Co., Kyoto, Japan).

The *E. coli* cell growth was monitored by OD_{600} using a DU730 spectrophotometer (Beckman-Coulter), and cell concentration was determined using a coefficient of 0.39 g/L per OD_{600}. Before measuring OD_{600}, calcium carbonate in fermentation broth was solubilized by 0.2 M HCl. PDC concentration in the fermentation media was determined by using high-performance liquid chromatography (HPLC) using a UV/RI detector (SPD-20A / RID-10A, Shimadzu Co., Kyoto, Japan) and ICSep ICE-ION-300 column. The operating conditions were a flow rate of 0.4 mL min^{-1} of 0.0085 M H_2SO_4 mobile phase with a column temperature of 70 °C.

3.3 Results and Discussion

3.3.1 Carbohydrate content of microalgae and glucose concentration in the hydrolysate

Before the fermentation for PDC production by the recombinant *E. coli*, the carbohydrate content of microalgae *C. emersonii*, *Parachlorella kessleri*, and *Chlorella vulgaris* and glucose concentration in each acid hydrolysate were evaluated. It was found that the carbohydrate content was 5.87 wt. % in the *C. emersonii* obtained *via* proliferation culture in TAP medium and subsequent starch accumulation culture in S-TAP medium. The culture of microalgae and freeze-dried microalgae are illustrated in **Fig. 3.6** and **3.7**. The glucose concentration and carbohydrate content of microalgae were shown in **Fig. 3.8**. The carbohydrate content was calculated by the following equation.

$$\text{Carbohydrate content (wt. \%)} = \frac{\text{Amount of glucose in algae hydrolysate (g)}}{\text{Amount of dried algae for acid hydrolysis (g)}} \times 100$$

The algae hydrolysate obtained after the acid-hydrolysis of 500 mg dried algae and neutralization contained glucose 16.3 g/L, total organic carbon (19.1 g/L), total nitrogen (4.1 g/L), Mn^{2+} (53.9 mg/L), and Fe^{2+} (0.38 mg/L). This algae hydrolysate was used for the following experiment of microbial fermentation for PDC production by the recombinant *E. coli* (original strain). The photo of algae hydrolysate containing 16.3 g/L glucose was illustrated in **Fig. 3.9**.

Fig. 3.6 Photo of microalgae cultivation

Fig. 3.7 Photo of freeze-dried microalgae

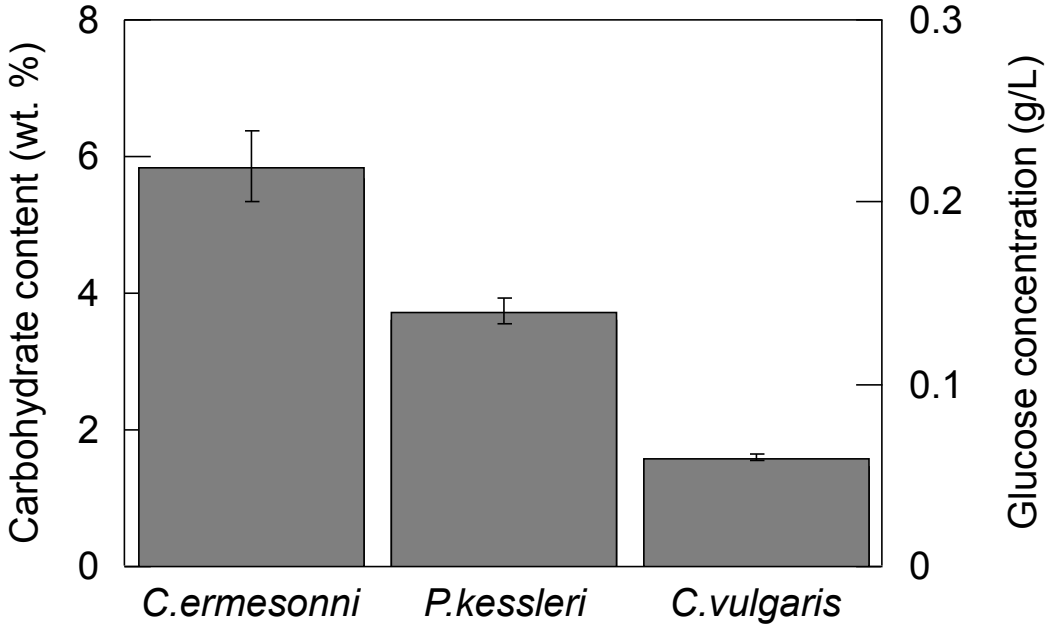

Fig. 3.8 Carbohydrate content and glucose concentration of green microalgae

Fig. 3.9 Photo of algae hydrolysate containing 16 g/L glucose

3.3.2 Effect of glucose source on cell growth and PDC production

Firstly, to examine the availability of algae hydrolysate as a substrate for PDC production, the microbial fermentation was carried out using the algae hydrolysate medium or the pure glucose medium (control), where the medium was supplemented with ×1 nutrients and the initial glucose concentration was adjusted to be 2 g/L. As shown in **Fig. 3.10 A**, glucose was consumed with elapsed time, being depleted at 6 h irrespective of the media. As shown in **Fig. 3.10 B**, in the case of the pure glucose medium, the cell concentration reached a saturated value of 1.8 g/L just after the glucose depletion at 6 h. On the contrary, in the case of the algae hydrolysate medium, the cell concentration continued to increase even after the glucose depletion at 6 h, reaching a maximum value of 5.5 g/L at 36 h. As shown in **Fig. 3.10 C**, in the case of the pure glucose medium, the PDC concentration reached 0.19 g/L at 24 h. On the other hand, in the case of the algae hydrolysate medium, the PDC concentration reached 0.43 g/L at 24 h, which was approximately 2.3-fold higher than that in pure glucose medium. The yield of PDC in the pure glucose medium was 8.6 %, whereas the value was increased to 20.1 % in the algae hydrolysate medium. It was considered that the nutrients in the algae hydrolysate (such as amino acids) were used as carbon sources for cell growth and PDC synthesis, leading to the higher cell and PDC concentration in the case of the algae hydrolysate medium. Also, protein, vitamin, etc. derived from algae in algae hydrolysate can enhance PDC production. Thus, the results showed that algae hydrolysate could be an effective fermentative substrate for PDC production.

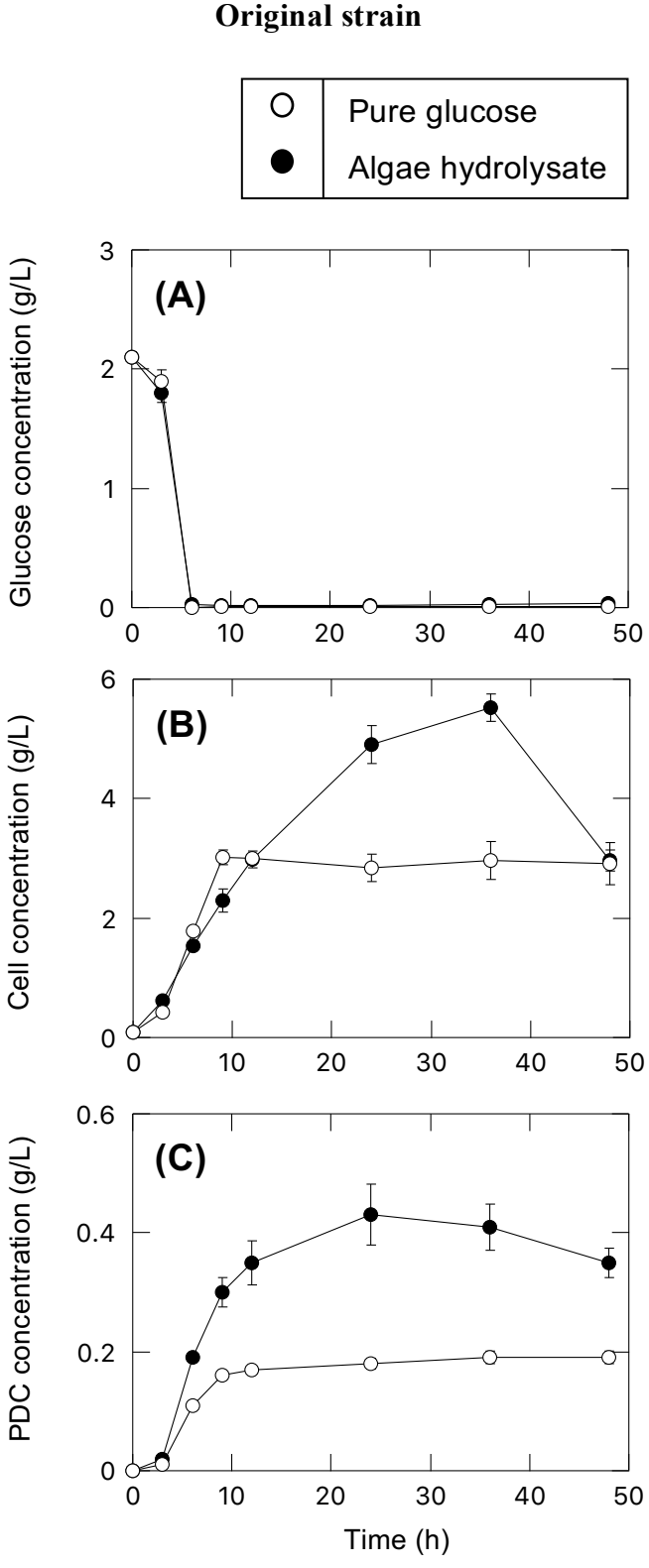

Fig. 3.10 Time courses of (A) glucose, (B) cell, and (C) PDC concentration during fermentation using pure glucose and algae hydrolysate media supplemented with ×1 nutrients. Initial glucose concentration in the fermentation media was set to be 2 g/L. The error bars indicate the standard deviation from three independent experiments.

3.3.3 Effect of additional nutrient amount on cell growth and PDC production

Next, to examine the possibility of PDC production from the algae hydrolysate medium without additional nutrient, the microbial fermentation was carried out using the algae hydrolysate medium or the pure glucose medium (control) with the addition of ×1, ×1/2, ×1/4, ×1/8, and ×0 nutrients, where the initial glucose concentration was adjusted to be 2 g/L. As shown in **Fig. 3.11**, the reduction of nutrient from ×1 nutrient to ×0 nutrient resulted in deterioration of glucose consumption, cell growth and PDC production, irrespective of the medium used. And, at all nutrition levels from ×1 nutrient to ×0 nutrient, the use of algae hydrolysate medium resulted in the higher cell and PDC concentrations compared with the case of the pure glucose medium. In concrete, almost no glucose consumption was seen in the case of the pure glucose medium with ×0 nutrient (**Fig. 3.11 A**); on the other hand, glucose was consumed and depleted at 12 h in the case of the algae hydrolysate medium even with ×0 nutrient (**Fig. 3.11 B**). No cell growth was confirmed in the case of the pure glucose medium with ×0 nutrient (**Fig. 3.11 C**); on the other hand, the cell concentration reached 1.0 g/L in the case of the algae hydrolysate medium even with ×0 nutrient (**Fig. 3.11 D**), and the value was roughly comparable to that in the case of the pure glucose medium with ×1/2 - ×1/4 nutrient.

Almost no PDC production was seen in case of the pure glucose medium with ×0 nutrient (**Fig. 3.11 E**); on the other hand, the PDC concentration reached 0.17 g/L at 48 h in the case of the algae hydrolysate medium even with ×0 nutrient (**Fig. 3.11 F**), and the value was almost comparable with that in the pure glucose medium with ×1 nutrient. These results indicate that the algae hydrolysate alone could be used as fermentation medium even without addition of nutrient. It is considered that the algae hydrolysate medium alone is considered to contain substantial nutrients of carbon and nitrogen sources for glucose consumption, cell growth and PDC production.

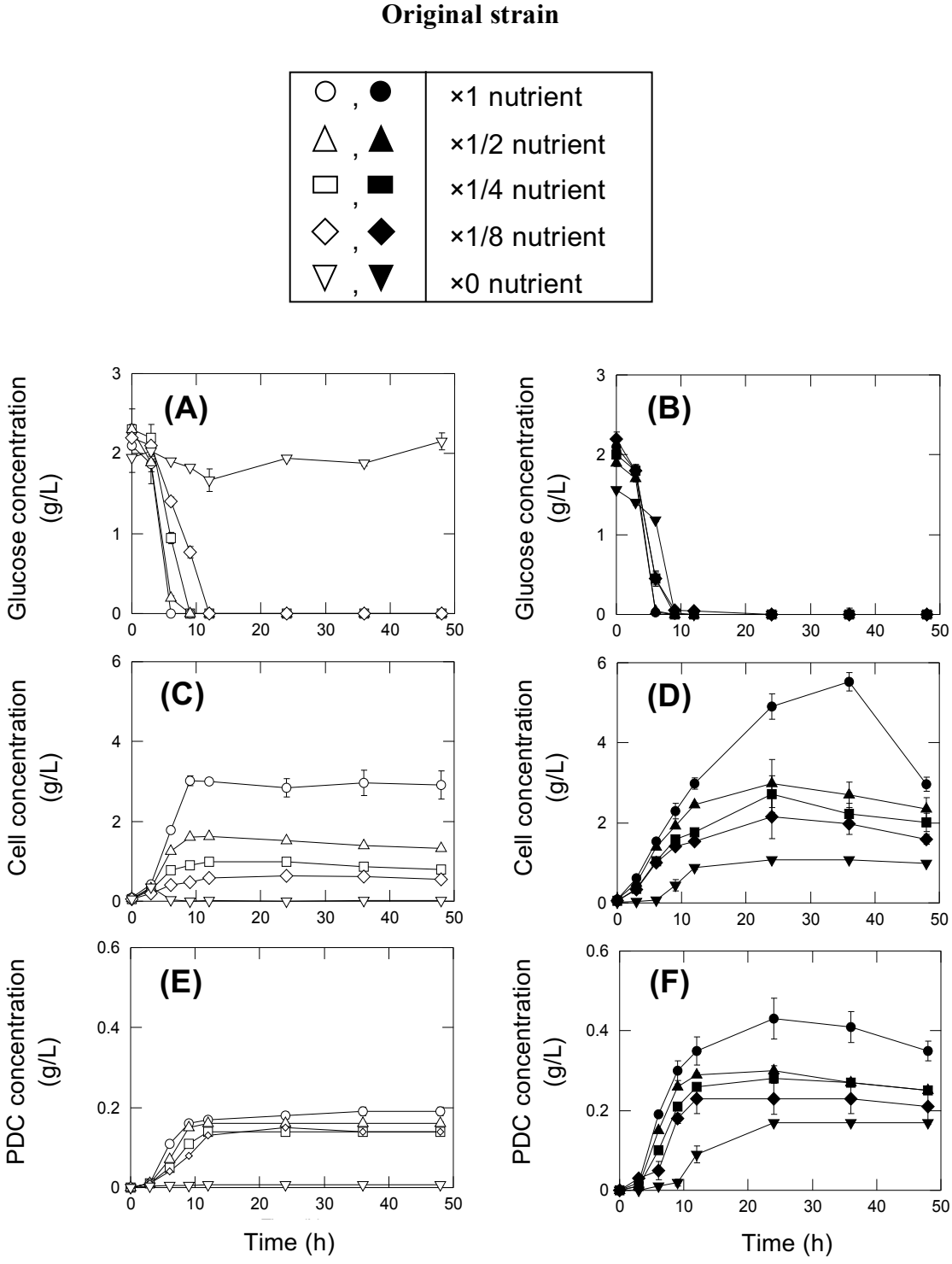

Fig. 3.11 Time courses of (A and B) glucose, (C and D) cell, and (E and F) PDC concentration during fermentation using pure glucose and algae hydrolysate media supplemented with reduced amount of nutrients. (A, C, and E) Pure glucose medium and (B, D, and F) algae hydrolysate medium. Initial glucose concentration in the fermentation media was set to be 2 g/L. The error bars indicate the standard deviation from three independent experiments.

To examine the detailed nutrient situation in the algae hydrolysate medium alone, TOC and TN in the medium were measured during the microbial fermentation using the algae hydrolysate medium or the pure glucose medium (control) with the addition of ×1, ×1/2, ×1/4, ×1/8, and ×0 nutrients, where the initial glucose concentration was adjusted to be 2 g/L. As shown in **Fig. 3.12**, the reduction of nutrient from ×1 nutrient to ×0 nutrient resulted in the reduction of initial TOC and TN concentration, irrespective of the medium used. And, at all nutrition levels from ×1 nutrient to ×0 nutrient, the use of algae hydrolysate medium resulted in the higher TOC and TN concentrations compared with the case of the pure glucose medium. Concretely, the time courses of TOC and TN concentration in the case of the algae hydrolysate medium with ×0 nutrient were roughly overlapped with those in the case of the pure glucose medium with ×1/2 nutrient. These tendencies are roughly consistent with the phenomena observed in **Fig. 3.11**, that is, cell growth and PDC production in the case of the algae hydrolysate medium with ×0 nutrient were comparable with those in the case of the pure glucose medium with ×1/2 nutrient. These results also indicate that the algae hydrolysate alone contained substantial nutrients of carbon and nitrogen sources. However, cell growth and PDC production was stopped after 24 h of the fermentation using the algae hydrolysate medium with ×0 nutrient (**Fig. 3.11**) even though the TOC and TN were not depleted (**Fig. 3.12**), indicating that other nutrients (such as trace elements) were depleted in advance of carbon and nitrogen.

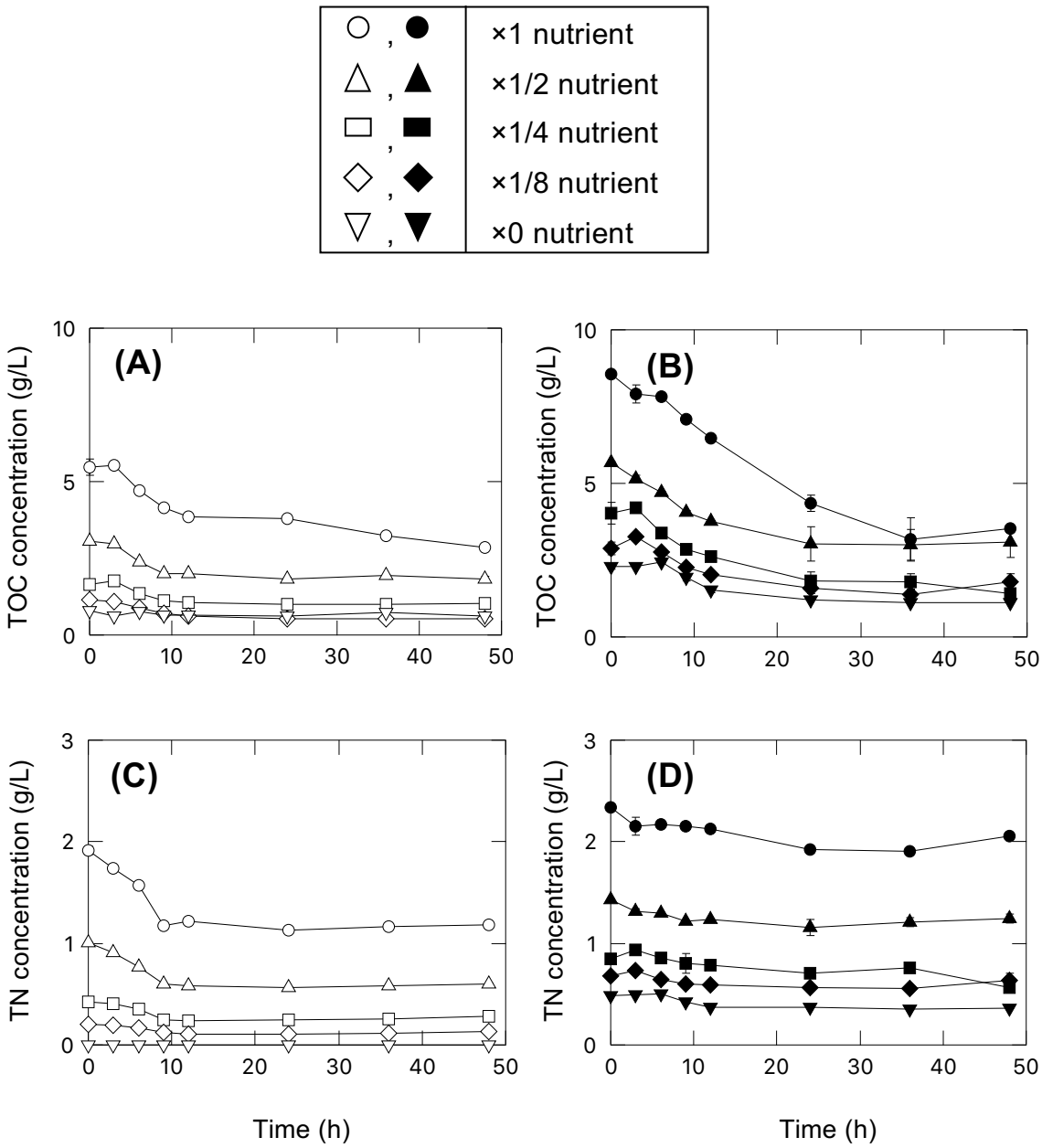

Fig. 3.12 Time courses of (A and B) TOC and (C and D) TN concentration during fermentation using pure glucose and algae hydrolysate media supplemented with the reduced amount of nutrients. (A and C) Pure glucose medium and (B and D) algae hydrolysate medium. Initial glucose concentration in the fermentation media was set to be 2 g/L. The error bars indicate the standard deviation from three independent experiments.

Trace elements are required for various intracellular enzymatic reactions involved in cell growth and PDC production. For example, Mn^{2+} is required for the activity of 3-dehydroshikimate dehydratase and Fe^{2+} is required in the protocatechuate 4,5-dioxygenase in PDC biosynthesis pathway. The Mn^{2+} and Fe^{2+} concentrations in the pure glucose medium with ×1 nutrient were lower than the minimum detectable limit (< 0.1 mg/L). In contrast, the algae hydrolysate medium contained more Mn^{2+} (10.6 mg/L) but Fe^{2+} was below the minimal detectable limit. To verify whether the level of Mn^{2+} affected the accelerated cell growth and PDC production observed in the algae hydrolysate medium, we added Mn^{2+} in the pure glucose medium to be set 10 mg/L. However, the cell growth and PDC concentration were not altered by the addition of Mn^{2+} (data not shown), implying that the accelerated PDC production was attributable to other nutrients or metals.

PDC productivity of actual plant microalgae supplemented with different nutrient level by original strain is shown in **Table 3.1**. PDC yield (mol. %) of algae hydrolysate medium supplemented with ×1 nutrient is 20.1 %. For the case of pure glucose medium with ×1 nutrient medium, PDC yield is 8.3 %. When additional nutrient was reduced by ×1/2, PDC yield from algae hydrolysate medium was 16.0 % and from pure glucose medium, PDC yield was 7.1 %. When pure glucose medium was not supplemented with additional nutrient, the yield of PDC was undeterminable. But, for the case of algae hydrolysate with no additional nutrient, the yield of PDC was 10.8 %. It was higher than PDC yield (8.3 %) from pure glucose medium with ×1 nutrient.

Table 3.1 PDC productivity of microalgae supplemented with different level of nutrient by original strain

Operational parameter		PDC yield (mol. %)
① Substrate	② Nutrient	
Pure glucose	×1 nutrient	8.3
Diluted algae hydrolysate (2 g/L glucose)		20.1
Pure glucose	×1/2 nutrient	7.1
Diluted algae hydrolysate (2 g/L glucose)		16.0
Pure glucose	×1/4 nutrient	6.3
Diluted algae hydrolysate (2 g/L glucose)		13.4
Pure glucose	×1/8 nutrient	6.7
Diluted algae hydrolysate (2 g/L glucose)		10.5
Pure glucose	Not added	Undeterminable
Diluted algae hydrolysate (2 g/L glucose)		10.8

3.3.4 High production of PDC from the algae hydrolysate without additional nutrient

Finally, to achieve the maximum PDC titer from the algae hydrolysate medium without additional nutrient, the microbial fermentation was carried out using the non-diluted original algae hydrolysate which contained 16 g/L glucose. As a comparison, the original hydrolysate was diluted to adjust the glucose concentration to 2, 5 and 10 g/L. As shown in **Fig. 3.13 A**, in the cases of algae hydrolysate medium containing 2, 5 and 10 g/L, the glucose concentration decreased with elapsed time and depleted completely within 24 h. However, in the case of the non-diluted algae hydrolysate medium containing 16 g/L glucose, the glucose concentration did not decrease throughout the examined culture time for 48 h. As shown in **Fig. 3.13 B**, in the cases of algae hydrolysate medium containing 2, 5 and 10 g/L, cell concentration reached 0.98 g/L, 3.2 g/L, and 6.8 g/L at 48 h, respectively. However, no substantial cell growth was confirmed in the case of the non-diluted algae hydrolysate medium containing 16 g/L glucose.

As shown in **Fig. 3.13 C**, in the cases of algae hydrolysate medium containing 2, 5 and 10 g/L glucose, PDC concentration reached 0.17 g/L, 0.71 g/L, and 1.22 g/L at 24 h, respectively. However, PDC production was only 0.01 g/L at 24 h in the case of the non-diluted algae hydrolysate medium containing 16 g/L glucose. PDC productivity of actual plant microalgae with no additional nutrient by original strain is summarized in **Table 3.2**. The yield of PDC was 10.8 %, 12.4 %, 16.1 % for the cases of algae hydrolysate medium containing 2, 5, 10 g/L glucose, respectively (PDC yield was not determinable in the case of algae hydrolysate medium containing 16 g/L glucose).

Thus, in the present study, the highest PDC titer was 1.22 g/L with a yield of 16.1 % obtained at 24 h of the fermentation using the diluted algae hydrolysate medium containing 10 g/L glucose without addition of nutrients. Algae hydrolysate contained enough nutrients for *E. coli* growth without exogenous nutrients, as shown in **Fig. 3.13**. However, when the hydrolysate was non-diluted, some nutrients (e.g. carbon, NH_4^+, and unknown substances) other than glucose might be too excessive to inhibit glucose consumption, cell growth, and PDC production.

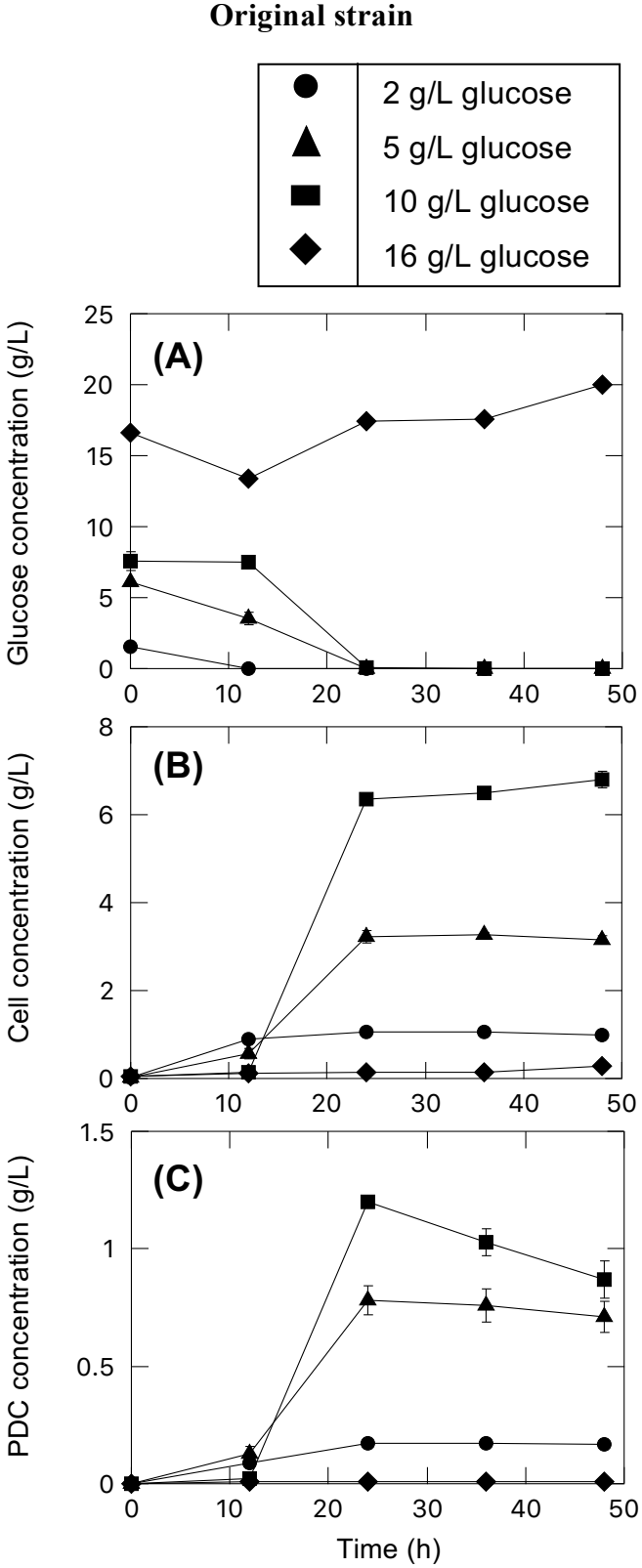

Fig. 3.13 Time courses of (A) glucose, (B) cell, and (C) PDC concentration during fermentation using algae hydrolysate media containing different initial glucose concentration, without supplementation of nutrients. The error bars indicate the standard deviation from three independent experiments.

Table 3.2 PDC productivity of microalgae by original strain

Operational parameter ① Substrate	PDC yield (mol. %)
Diluted algae hydrolysate (2 g/L glucose)	10.8
Diluted algae hydrolysate (5 g/L glucose)	12.4
Diluted algae hydrolysate (10 g/L glucose)	16.1
Diluted algae hydrolysate (16 g/L glucose)	Undeterminable

3.4 Summary

The possibility of algae hydrolysate as a fermentative substrate for PDC production by recombinant *E. coli* was investigated. From the obtained results, the following conclusions can be drawn.

(1) *Chlorella emersonni* has the higher carbohydrate content than the other two microalgae (*Parachlorella kessleri* and *Chlorella vulgaris*).

(2) The yield of PDC (mol.%) from diluted algae hydrolysate containing 2 g/L glucose supplemented with tryptone and yeast extract medium was 20.1 % (0.43 g/L).

(3) The yield of PDC (mol.%) from diluted algae hydrolysate containing 2 g/L glucose without additional nutrient medium was 10.8 % (0.17 g/L). The highest PDC concentration (1.2 g/L) with the yield of 10.8 % was obtained from diluted algae hydrolysate containing 10 g/L glucose with no additional nutrient medium.

(4) When non-diluted algae hydrolysate without additional nutrient was used as a fermentative substrate, no cell concentration and PDC production were confirmed. It was considered that in non-diluted algae hydrolysate, excess carbon, NH_4^+, and other unknown substances might inhibit cell growth and PDC production.

(5) Diluted algae hydrolysate alone can be used as effective fermentation substrate for PDC production. Protein, vitamin, and trace elements (other than Mn^{2+}, Fe^{2+}) derived from algae can support for enhancing PDC production. It can be saved using chemical for additional nutrient and for preparation of basal medium.

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General Conclusion

The production of 2-pyrone 4,6-dicarboxylic acid by recombinant *E. coli* was investigated to use as a bio-based plastic monomer. The various biomass feedstocks were used as the fermentative substrates. Furthermore, the efficiency of PDC producing recombinant *E. coli* with minor modification was studied.

In Chapter 1, PDC production process was investigated using glycerol, the by-product of biodiesel, as a fermentative substrate. In the enzymatic glycerol (via transesterification process in the presence of enzyme), the content of glycerol was 82.3 wt.%. The retained components were methanol, fatty acid methyl ester (biodiesel), water and other substances. Total fatty acid might contain in the composition of other substances. In the alkali glycerol (via transesterification process in the presence of alkali), the content of glycerol was 56.5 wt.%. The retained components were total fatty acid and other substances. Methanol, fatty acid methyl ester (biodiesel), and water might contain in the composition of other substances. For the fermentation process, M-9 medium and yeast extract were used as the basal medium and additional nutrient, respectively. The initial glycerol concentration was set to be 20 g/L. The yield of PDC (mol.%) from enzymatic glycerol and alkali glycerol media by the original strain were 4.9 % (1.9 g/L) and 4.1 % (1.6 g/L). It was considered that the impurities in both glycerol media did not inhibit cell growth and PDC production. Even though 40.1 wt.% of total fatty acid contained the alkali glycerol, it did not inhibit fermentation microorganism. The inconsiderable different from PDC yield was supposed due to the purity of glycerol. The yield of PDC (mol.%) from enzymatic glycerol and alkali glycerol media by the modified strain were 4.7 % (1.9 g/L) and 3.0 % (1.6 g/L). It showed that PDC yield was the same for the original strain and the modified strain. The reason was glycerol uptake pathway in *E. coli* does not relate to PTS. Therefore, even PTS was deleted to terminate some

reactions for saving more phosphoenolpyruvate (PEP) molecules to obtain more PDC molecules, it did not effect on glycerol substrate for PDC production.

In Chapter 2, PDC production process was investigated using bagasse, the by-product of sugar. Bagasse was pretreated with choline acetate (ChOAc) and then hydrolyzed by cellulase. Choline acetate has less inhibitory to cellulase and the hydrolysate solution obtained by cellulase contained less amount of inhibitory compounds to fermentative microorganisms. Pretreatment and hydrolysis process of bagasse was one of the critical processes because the composition of hydrolysate solution can vary depending on the pretreatment and hydrolysis method used. The enzyme-hydrolyzed solution from bagasse (defined as bagasse hydrolysate in the present study) was used as a fermentative substrate. The main components in bagasse hydrolysate solution were glucose and xylose. M-9 medium was used as the basal medium and to evaluate the effect of additional nutrient, yeast extract was used. The initial sugar concentration was set to be 20 g/L. By the original strain, yield PDC (mol.%) of medium with yeast extract was 30.9 % (4.3 g/L) and that of medium without yeast extract was 19.7 % (2.7 g/L). It was supposed that bagasse-hydrolysate could be used as a fermentative substrate with basal medium and additional nutrient did not need to use for PDC production. It was considered that amino acid and nucleic acid derived from the decomposition of cellulose or hemicellulose could support to enhance PDC productivity. By the modified strain, yield PDC (mol.%) of medium with yeast extract was 38.1 % (5.4 g/L) and that of medium without yeast extract was 31.7 % (4.8 g/L). PDC productivity was enhanced by the modified strain than the original strain. The reason was glucose uptake pathway in *E. coli* relates to PTS. Therefore, when PTS was deleted to terminate some reactions for saving more phosphoenolpyruvate (PEP) molecules to obtain more PDC molecules, it significantly effects on bagasse hydrolysate substrate for PDC production.

In Chapter 3, PDC production process was investigated using microalgae, actual plant biomass. Microalgae were cultivated and accumulated starch under the specific condition. Starch accumulated microalgae were acid-hydrolyzed. The obtained hydrolysate solution (algae hydrolysate) was used as the fermentative substrate. This algae hydrolysate contained glucose 16.3 g/L, total organic carbon (19.1 g/L), total nitrogen (4.1 g/L), Mn^{2+} (53.9 mg/L), and Fe^{2+} (0.38 mg/L). For the fermentation of algae hydrolysate by recombinant *E. coli*, no basal medium was used. To evaluate the effect of the additional nutrient on algae hydrolysate for PDC production, tryptone and yeast extract were used as the additional nutrient. The yield of PDC (mol.%) from diluted algae hydrolysate containing 2 g/L of glucose supplemented with tryptone and yeast extract medium was 20.1 % (0.43 g/L). To obtain high PDC concentration from algae hydrolysate without using additional nutrient, non-diluted algae hydrolysate containing 16 g/L glucose compared to diluted algae hydrolysate containing 2, 5, and 10 g/L glucose were used. For non-diluted algae hydrolysate, no cell growth was confirmed and PDC concentration was lower than 0.1 g/L. But for the case of algae hydrolysate containing 2, 5, and 10 g/L glucose, the yield of PDC (mol. %) were 10.8 % (0.17 g/L), 12.4 % (0.71 g/L), and 16.1 % (1.22 g/L), respectively. It was considered that in non-diluted algae hydrolysate, some nutrients such as carbon, NH_4^+, and other unknown substances other than glucose were might excess to inhibit cell growth and PDC production. Also, it was supposed that vitamin, protein, and trace elements (other than Mn^{2+} and Fe^{2+}) derived from algae in algae hydrolysate can support to enhance cell growth and PDC production.

In the present study, we investigated the possibility of different biomass feedstocks for the production of bio-based plastic monomer (PDC). By using algae hydrolysate as fermentative substrate for PDC production, it did not need to use additional nutrient and even basal medium. Therefore, amount of additional nutrient and other chemicals used for preparation of medium can be saved. Finally, it can be concluded that

among three different feedstocks, microalgae have the highest potential to use as the effective fermentation substrate and economically viable process for PDC production by recombinant *E. coli*.

List of Publication

1. Application of microalgae hydrolysate as a fermentation medium for microbial production of 2-pyrone 4,6-dicarboxylic acid.

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